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**Cyclin-dependent kinase (CDK) inhibitor drugs induce apoptosis in human neutrophils through regulation of critical survival proteins.**

**Nicola Amy Riley**

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## **Declaration**

I hereby declare that the data presented in this thesis are the result of my own work carried out under the supervision of Professors Adriano G. Rossi and Chris Haslett at the University of Edinburgh. This thesis has been completed entirely by myself and has not previously been submitted for any other degree or qualification.

Nicola Amy Riley

Edinburgh, August 2011

## **Abstract**

Neutrophil apoptosis is an important process contributing to the resolution of inflammation. This is because it allows the neutrophil membrane to remain intact preventing its potentially histotoxic contents from being released into the extra-cellular milieu, a process that can contribute to the exacerbation of many inflammatory disorders such as rheumatoid arthritis. When considering the life-span of a neutrophil and how it can be augmented by various inflammatory mediators to allow it to carry out its essential protective role in the body's innate immune defences it is also important to consider how to terminate this process when the inflammatory insult has been dealt with or when the system goes awry. It is this information that we believe may hold the key to developing novel anti-inflammatory therapies. Through exploitation of the mechanisms controlling neutrophil apoptosis, it may be possible to selectively target these cells to enter apoptosis, and therefore help aid the process of resolution, especially if used in conjunction with treatments that up-regulate phagocytosis of apoptotic cells. This is important given that the main treatment for disorders of the inflammatory response are glucocorticoids, which whilst proven to be a powerful treatment for eosinophil based diseases such as asthma where they increase eosinophil apoptosis in conjunction with enhancing phagocytic clearance of apoptotic cells, glucocorticoids have been found to have the converse affect on neutrophils, actually serving to prolong their life-span potentially exacerbating the condition. Furthermore, it has been previously shown that the transcription factor nuclear factor kappa B (NF- $\kappa$ B) plays a pivotal role in neutrophil apoptosis, becoming activated by inflammatory agents such as lipopolysaccharide (LPS) and tumour necrosis factor-alpha (TNF- $\alpha$ ). NF- $\kappa$ B activation results in the transcription of many pro-inflammatory agents and anti-apoptotic proteins such as X-linked inhibitor of apoptosis (X-IAP) increasing the life-span of the neutrophil. Interestingly, it has also been demonstrated that key neutrophil survival proteins such as myeloid cell leukemia-1 (Mcl-1) are not directly regulated by NF- $\kappa$ B activation. Therefore it is because of the aforementioned reasons that I have chosen to investigate further neutrophil apoptosis including the role played by NF- $\kappa$ B. Thus, I have investigated the hypothesis that NF- $\kappa$ B-dependent and independent survival proteins critically regulate the rates of neutrophil apoptosis and that newly identified pro-apoptotic agents such as the

cyclin-dependent kinase (CDK) inhibitor, R-roscovitine interferes with the expression of such survival proteins.

It has been previously found by myself and others in our laboratory during the course of this thesis that cyclin dependent kinase inhibitor (CDKi) drugs such as R-roscovitine are powerful novel anti-inflammatory agents with the ability to up-regulate rates of neutrophil apoptosis *in vitro* and influence the resolution of neutrophilic inflammation *in vivo*. Whilst the exact mechanism of CDK inhibitor drugs on neutrophil apoptosis remains elusive, work shown in this thesis demonstrates that R-roscovitine has the ability to over-ride powerful anti-apoptotic signals from pro-inflammatory agents such as granulocyte-macrophage colony stimulating factor (GM-CSF) and LPS causing the neutrophils to enter apoptosis. Furthermore, it has been found that R-roscovitine causes a decrease in levels of the anti-apoptotic protein Mcl-1 in as little as 2h and that it prevents the maintenance / protective effect that GM-CSF has on the Mcl-1 protein levels. In addition R-roscovitine may also reduce levels of the NF- $\kappa$ B regulated protein X-IAP. The effect of R-roscovitine on X-IAP was investigated further using an X-IAP HIV-tat construct, though results from this remain inconclusive. This is because although the X-IAP construct appeared to be extending neutrophil longevity, it was discovered that LPS contamination of the construct had occurred which could therefore pose an alternative explanation for the increase in neutrophil life-span.

As X-IAP, TNF- $\alpha$  and LPS are all regulated by NF- $\kappa$ B and given that NF- $\kappa$ B is already known to be a key player in neutrophil biology, the effects of R-roscovitine on this important transcription factor were investigated. It was discovered that R-roscovitine does not directly activate NF- $\kappa$ B, since this CDK inhibitor drug does not cause degradation and loss of the cytoplasmic inhibitor of NF- $\kappa$ B, I $\kappa$ B $\alpha$ . This lack of NF- $\kappa$ B activation was confirmed since R-roscovitine did not mobilize the NF- $\kappa$ B subunit, p65, from the cytoplasm to the nucleus. Furthermore, R-roscovitine (unlike the NF- $\kappa$ B inhibitor gliotoxin) does not interfere with the ability of LPS or TNF- $\alpha$  to activate NF- $\kappa$ B. Therefore these results indicate that NF- $\kappa$ B is unlikely to be one of the pathways directly influenced

by R-roscovitine to induce apoptosis, although this does not rule out the involvement of NF- $\kappa$ B at a later stage.

When considering a reagent for possible use as a novel anti-inflammatory agent I think it is important to assess what effects it has on the activation state of the neutrophil. Therefore the effects of R-roscovitine on the activation markers CD62L, CD11b and shape change were assessed. It was found that R-roscovitine alone did not cause any significant neutrophil activation as measured using the parameters stated above. Importantly, it was also found that R-roscovitine did not interfere with the activation states induced by the inflammatory mediators GM-CSF, LPS, TNF- $\alpha$  or leukotriene B<sub>4</sub> (LTB<sub>4</sub>).

Another important consideration is the effect of R-roscovitine on the removal of apoptotic cells by macrophage phagocytosis. Results demonstrated that pre-treatment of macrophages with R-roscovitine did not augment their uptake of apoptotic neutrophils. In addition R-roscovitine did not detrimentally affect the increase in phagocytosis that results from macrophage treatment with the synthetic glucocorticoid dexamethasone.

The data presented in this thesis suggest that CDK inhibitor drugs such as R-roscovitine are novel powerful pro-apoptotic agents for neutrophils with the ability to over-ride anti-apoptotic signals from multiple pro-inflammatory mediators. It has been discovered that R-roscovitine causes a reduction in one of the neutrophil's most prominent anti-apoptotic proteins (Mcl-1) whilst not altering the activation state of the neutrophil and furthermore it does not interfere with the uptake of apoptotic neutrophils by macrophages or result in any alteration to the increase in phagocytosis caused by treatment with dexamethasone. In conclusion, CDK inhibitor drugs such as R-roscovitine have the potential to be promising candidates for novel anti-inflammatory agents with the ability to selectively target neutrophil apoptosis whilst not interfering with steroid induced up-regulation of phagocytosis, therefore allowing a two pronged attack to help treat neutrophil based inflammatory disorders.

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### **Index of publications arising from this thesis**

- A.G. Rossi, D.A. Sawatzky, A. Walker, C. Ward, T.A. Sheldrake, **N.A. Riley**, A. Caldicott, M. Martinez-Losa, T.R. Walker, R. Duffin, M. Gray, E. Crescenzi, H.J. Brady, J.S. Savill, I. Dransfield, C. Haslett. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. Nat Med. 2006 Sep;12 (9):1056-64. Epub 2006 Sep 3.
- **N.A. Riley**, C. Ward, D.A. Sawatzky, T.A. Sheldrake, I. Dransfield, C. Haslett and A.G. Rossi. Granulocyte Apoptosis and Macrophage Clearance of Apoptotic Cells as Targets for Pharmacological Intervention in Inflammatory Diseases. Anti-Inflammatory and Anti-Allergy Agents in Medicinal Chemistry. 2006, volume 5, 3-13
- Hallet JM, Leitch AE, **Riley NA**, Duffin R, Haslett C, Rossi AG. Novel pharmacological strategies for driving inflammatory cell apoptosis and enhancing the resolution of inflammation. Trends Pharmacol Sci 2008 May; 29(5):250-7. Epub 2008 Apr 11. Review.
- Leitch AE, **Riley NA**, Sheldrake TA, Festa M, Fox S, Duffin R, Haslett C, Rossi AG. The cyclin-dependent kinase inhibitor R-roscovitine down-regulates Mcl-1 to override pro-inflammatory signalling and drive neutrophil apoptosis. Eur J Immunol. 2010 Apr; 40(4):1127-38.

### **Abstracts**

- ECDO 14<sup>th</sup> Euroconference on Apoptosis, September 2006 in Cagliari, Sardinia. Poster Abstract P-195, Life and death of a neutrophil: The role of NF- $\kappa$ B dependent and independent survival proteins in neutrophil apoptosis. **Nicola A. Riley**, Christopher Haslett and Adriano G. Rossi.



## **Abbreviations**

AEBSF	4-(2-Aminoethyl) Benzenesulfonyl Fluoride Hydrochloride
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
APC	Allophycocyanin Conjugate
APS	Adenosine 5'-Phosphosulfate sodium salt
BAFF	B-cell Activating Factor
Bcl-2	B-cell lymphoma 2
C5a	Complement component 5a
cAMP	Cyclic Adenosine Monophosphate
CCR5	CC-chemokine Receptor 5
CDK	Cyclin-Dependent Kinase
CDKi	Cyclin-Dependent Kinase Inhibitor
cDNA	Complementary DNA
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
COMMD1	Copper Metabolism Domain Containing 1
COPD	Chronic Obstructive Pulmonary Disease
CR1	Complement Receptor 1
CREB	cAMP Response Element-Binding
CTD	Carboxyl-Terminal Domain
CX <sub>3</sub> CL <sub>1</sub>	CX <sub>3</sub> C-chemokine ligand 1
CX <sub>3</sub> CR <sub>1</sub>	CX <sub>3</sub> C-chemokine ligand receptor 1
dbcAMP	Dibutyl Cyclic Adenosine 3',5'-Monophosphate
DED	Death Effector Domain
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide Triphosphate
DSIF	DRB Sensitivity Inducing Factor
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence

EDTA	Ethylenediaminetetraacetic Acid
EMAP II	Endothelial Monocytes-Activating Polypeptide II
ERK	Extracellular signal-Regulated Kinases
ESL-1	E-selectin Ligand-1
Ets	E-twenty six
FADD	Fas-Associated Death Domain
FITC	Fluorescein Isothiocyanate
fMLP	Formylmethionyl Leucyl Phenylalanine
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GR	Glucocorticoid Receptor
GSK-3 $\beta$	Glycogen Synthase Kinase 3 beta
GTP	Guanosine Triphosphate
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia Inducible Factors
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis
ICAM-1	Inter-Cellular Adhesion Molecule-1
ICAM-2	Inter-Cellular Adhesion Molecule-2
IFN- $\gamma$	Interferon gamma
I $\kappa$ B $\alpha$	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKK	I kappa Kinase
IL	Interleukin
IMDM	Iscoe's Modified Dulbecco's Media
JAMs	Junctional Adhesion Molecules
JNK	c-Jun NH <sub>2</sub> -terminal Kinase
LAL	Limulus Amoebocyte Lysate
LFA-1	Lymphocyte function-associated antigen 1
LPC	Lysophosphatidylcholine

LTβ	Lymphotoxin beta
LTB <sub>4</sub>	Leukotriene B <sub>4</sub>
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MAPK	Mitogen-Activated Protein Kinase
Mcl-1	Myeloid cell leukaemia differentiation protein
MEKK	Mitogen-activated protein kinase kinase kinase
MG-132	N-cbz-Leu-Leu-leucinal
MGF-E8	Milk-Fat Globule Epidermal Growth Factor 8
MIF	Migration Inhibitory Factor
MPO	Myeloperoxidase
mRNA	Messenger RNA
MULE	Mcl-1 Ubiquitin Ligase E3
NELF	Negative Elongation Factor
NF-κB	Nuclear Factor Kappa B
NIK	NFκB-Inducing Kinase
NP-40	Nonyl Phenoxypolyethoxylethanol
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
Oligo DT	Oligodeoxythymidylic acid
PAF	Platelet Activating Factor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PEST	proline, glutamic acid, serine, threonine rich domain
PG	Prostaglandin
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinases
PK	Protein Kinase
pNA	p-nitroaniline
PS	Phosphatidylserine

PSGL-1	P-selectin Glycoprotein Ligand-1
PTD	Protein Transduction Domain
PVDF	Polyvinylidene Fluoride
RAGE	Receptor for Advanced Glycation Endproducts
RANKL	Receptor activator of nuclear factor kappa B ligand
RING	Really Interesting New Gene
RIP	Receptor-Interacting Protein
RNA	Ribonucleic Acid
RNA Pol II	Ribonucleic Acid Polymerase II
RNase	Ribonuclease
ROS	Reactive Oxygen Species
R-roscovitine	(R)-2-[[9-(1-methylethyl)-6-[(phenylmethyl)amino]-9H-purin-2-yl]amino]-1-butanol
rtPCR	Reverse Transcription Polymerase Chain Reaction
S1P	Sphingosine-1 Phosphate
SAPK	Stress-Activated Protein Kinase
SCF	Stem Cell Factor
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis
STAT	Signal Transducer and Activator of Transcription
TAB1	TAK binding protein 1
TAK1	TGF- $\beta$ -activated kinase 1
Tat	Trans-Activator of Transcription
TBE	Tris/Borate/EDTA
tBid	Truncated Bid
TBS	Tris Buffered Saline
TEMED	Tetramethylethylenediamine
TGF $\beta$	Transforming Growth Factor- $\beta$
TNF- $\alpha$	Tumor Necrosis Factor-alpha
TNFR	Tumor Necrosis Factor Receptor
TRADD	TNFR-Associated Death Domain-containing proteins
TRAIL	Tumor Necrosis Related Apoptosis-Inducing Ligand

TRAILR	Tumor Necrosis Related Apoptosis-Inducing Ligand Receptor
VEGF	Vascular Endothelial Growth Factor
XIAP	X-Linked Inhibitor of Apoptosis
zVAD-fmk	Benzylocarbonyl-Val-Ala-Aspfluoromethylketone
15-epi-LXA4	15-epi-lipoxin A4

## **Table of Contents**

<b>DECLARATION</b>	<b>I</b>
<b>ABSTRACT</b>	<b>II</b>
<b>ACKNOWLEDGEMENTS</b>	<b>V</b>
<b>INDEX OF PUBLICATIONS ARISING FROM THIS THESIS</b>	<b>VI</b>
<b>ABBREVIATIONS</b>	<b>VII</b>
<b>TABLE OF CONTENTS</b>	<b>XII</b>
<b>FIGURE INDEX</b>	<b>XVII</b>
<b>CHAPTER 1</b>	<b>1</b>
<b>Introduction</b>	
<b>1.1 The Inflammatory Response</b>	<b>1</b>
<b>1.2 Haematopoiesis</b>	<b>2</b>
<b>1.3 Leucocytes</b>	<b>3</b>
<b>1.4 The neutrophil</b>	<b>3</b>
<b>1.5 Neutrophil recruitment to sites of inflammation</b>	<b>4</b>
1.5.1 Rolling	5
1.5.2 Adhesion	5
1.5.3 Diapedesis	6
1.5.4 Migration	7
<b>1.6 Neutrophil granules and Secretory vesicles</b>	<b>7</b>
<b>1.7 Monocytes</b>	<b>10</b>
<b>1.8 Macrophages</b>	<b>11</b>
<b>1.9 Apoptosis</b>	<b>12</b>
1.9.1 Structural and cellular changes occurring during granulocyte apoptosis	12
1.9.2 Apoptotic pathways	15
1.9.2.1 Extrinsic pathway	15
1.9.2.2 Intrinsic pathway	16
1.9.3 Cysteine-dependent aspartate-specific protease family (Caspases)	16
1.9.4 Cross talk between Intrinsic and Extrinsic pathways	17

<b>1.10 Neutrophil survival proteins</b>	<b>19</b>
1.10.1 Myeloid cell leukaemia differentiation protein (Mcl-1)	20
1.10.2 Mcl-1 function	22
1.10.3 Role of Mcl-1 in the neutrophil	23
1.10.4 X-linked Inhibitor of Apoptosis (X-IAP)	25
<b>1.11 Intracellular signaling affecting granulocyte survival</b>	<b>27</b>
1.11.1 NF- $\kappa$ B pathway and its role in neutrophil apoptosis	27
1.11.2 Phosphoinositide 3-kinase (PI3-K)	29
1.11.3 Mitogen Activated Protein Kinase (MAPK)	30
1.11.4 Cyclic Adenosine Mono Phosphate (cAMP)	31
1.11.5 Calcium	31
1.11.6 Glucocorticoids	32
1.11.7 Nitric oxide	33
1.11.8 Miscellaneous	33
<b>1.12 Macrophage removal of apoptotic granulocytes</b>	<b>34</b>
<b>1.13 Resolution of inflammation</b>	<b>36</b>
1.13.1 Resolution of inflammation and the role of the neutrophil	39
<b>1.14 Non resolving inflammation</b>	<b>41</b>
<b>1.15 Targets for pharmacological intervention</b>	<b>42</b>
<b>1.16 Therapeutic applications and drug discovery</b>	<b>43</b>
<b>1.17 Cyclin-dependent kinases</b>	<b>45</b>
1.17.1 The role of CDKs in granulocytes	47
1.17.2 The role of CDKs in transcription	48
<b>1.18 Pharmacological CDK inhibitors</b>	<b>51</b>
<b>1.19 Effects of R-roscovitine on neutrophils</b>	<b>51</b>
<b>1.20 Hypothesis and Aims</b>	<b>54</b>
 <b>CHAPTER 2</b>	 <b>55</b>
<b>Materials and Methods</b>	
<b>2.1 Reagents</b>	<b>55</b>
<b>2.2 Antibodies</b>	<b>55</b>

<b>2.3 Isolation of mononuclear and polymorphonuclear leucocytes from whole blood</b>	<b>56</b>
<b>2.4 Assessment of neutrophil purity and viability</b>	<b>59</b>
<b>2.5 Methods of assessing neutrophil apoptosis</b>	<b>61</b>
2.5.1 Cell morphology	61
2.5.2 Annexin V/Propidium Iodide (PI) staining	62
<b>2.6 Western Blotting for Mcl-1, X-IAP and IκBα</b>	<b>63</b>
2.6.1 Neutrophil lysis	63
2.6.2 Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE)	64
2.6.3 Western Blotting	65
<b>2.7 Immunohistochemistry and Confocal analysis</b>	<b>65</b>
2.7.1 Annexin V binding/PI staining	65
2.7.2 Phagocytosis assay for apoptotic neutrophils	66
2.7.2.1 Cell isolation and culture	66
2.7.2.2 Aging neutrophils	66
2.7.2.3 Phagocytosis	66
2.7.3 Assessment of CD62L, CD11b and Shape Change	67
<b>2.8 X-IAP HIV-tat</b>	<b>67</b>
2.8.1 Effects of X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 18 h	68
2.8.2 Effects of X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 4 h	68
2.8.3 X-IAP-tat construct effects on neutrophil shedding of CD62L and Shape Change	68
<b>2.9 Limulus Amoebocyte Lysate chromogenic endpoint Assay (LAL Assay)</b>	<b>69</b>
<b>2.10 RT-PCR for Mcl-1</b>	<b>69</b>
2.10.1 DNase treatment of RNA samples	70
2.10.2 Making cDNA from RNA	70
2.10.3 RT-PCR	70
2.10.4 PCR primers	71



2.10.5 1 % Agarose gel and running buffer	71
<b>2.11 Statistical Analysis</b>	<b>72</b>
<b>2.12 Densitometry</b>	<b>72</b>
 <b>CHAPTER 3 Regulation of neutrophil apoptosis by the CDK inhibitor R- roscovitine and its effects on subsequent macrophage phagocytosis.</b>	 <b>73</b>
<b>3.1 Introduction</b>	<b>73</b>
3.1.1 CDKs and the neutrophil	73
<b>3.2 Results</b>	<b>76</b>
3.2.1 Effect of TNF- $\alpha$ and the NF- $\kappa$ B inhibitor gliotoxin on neutrophil apoptosis at 2 h	76
3.2.2 Effect of R-roscovitine on neutrophil apoptosis <i>in vitro</i> at 4 h	78
3.2.3 Stimuli affecting neutrophil apoptosis at 20 h	81
3.2.4 Effect of R-roscovitine on markers of neutrophil activation	83
3.2.5 Effect of R-roscovitine on macrophage phagocytosis of apoptotic neutrophils <i>in vitro</i>	90
<b>3.3 Discussion</b>	<b>94</b>
 <b>CHAPTER 4 The effect of R-roscovitine on NF-<math>\kappa</math>B and NF-<math>\kappa</math>B dependent and independent survival proteins in neutrophils</b>	 <b>100</b>
<b>4.1 Introduction</b>	<b>100</b>
4.1.1 The NF- $\kappa$ B pathway	101
4.1.1.1 The Classical/Canonical NF- $\kappa$ B pathway	102
4.1.1.2 Alternative NF- $\kappa$ B pathway	103
4.1.2 NF- $\kappa$ B dependent and NF- $\kappa$ B independent survival proteins	104
4.1.2.1 X-IAP	106
4.1.2.2 Mcl-1	106
<b>4.2 Results</b>	<b>108</b>
4.2.1 Effect of mediators of neutrophil survival and R-roscovitine on I $\kappa$ B $\alpha$ in neutrophils	108
4.2.2 Effect of R-roscovitine on ability of LPS and TNF- $\alpha$ to activate NF- $\kappa$ B	112

4.2.3 Confocal micrographs of p65 translocation in neutrophils	114
4.2.4 Identification by Western Blotting of cIAP, X-IAP and A1 in human neutrophil lysates	116
4.2.5 Identification of Mcl-1 mRNA and protein in human neutrophil lysates	118
4.2.6 Effect of R-roscovitine on GM-SF induced neutrophil survival and Mcl-1	120
4.2.7 Effect of R-roscovitine on GM-CSF, TNF- $\alpha$ and LPS induced neutrophil survival and the anti-apoptotic protein Mcl-1	123
4.2.8 Effect of proteasome and caspase inhibitors on R-roscovitine and GM-CSF mediation of Mcl-1 levels in neutrophils	124
4.2.9 X-IAP levels in neutrophils	127
4.2.10 Effect of X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 18 h	130
4.2.11 Shape change in neutrophils treated with X-IAP-tat at 18 h	134
4.2.12 Effects of X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 4 h	137
4.2.13 Shape change in neutrophils treated with X-IAP-tat at 4 h	138
4.2.14 Effect of X-IAP-tat on CD62L expression and shape change in neutrophils	142
4.2.15 Limulus Amebocyte Lysate (LAL) test of the X-IAP-tat construct	145
<b>4.3 Discussion</b>	<b>147</b>
 <b>CHAPTER 5 Discussion</b>	 <b>153</b>
<b>5.1 Summary and general discussion</b>	<b>153</b>
<b>5.2 Limitations</b>	<b>158</b>
<b>5.3 Future directions</b>	<b>159</b>
<b>5.4 Concluding remarks</b>	<b>164</b>
 <b>6. REFERENCES</b>	 <b>165</b>
<b>7. PUBLICATIONS ARISING FROM THIS THESIS</b>	<b>184</b>

## **Table of Figures**

### **CHAPTER 1**

1.1 Haematopoiesis	2
1.2 Photomicrograph of freshly isolated human neutrophils	4
1.3 Table showing the main constituents of neutrophil granules and secretory vesicles	10
1.4 Factors influencing human granulocyte apoptosis	14
1.5 Diagrammatic representations of the extrinsic and intrinsic apoptotic pathways and cross talk between the two.	18
1.6 Diagrammatic representation of the two isoforms of Mcl-1	20
1.7 Structural representation of X-IAP	25
1.8 Interaction between leucocytes during resolution of inflammation	40
1.9 Schematic representation of CDK/cyclin complexes involved in the eukaryotic cell cycle	46
1.10 Diagrammatic representation of the role of CDK7 and CDK9 in eukaryotic transcription	50
1.11 Table showing the specificity of some of the purine analogue CDK inhibitors	51
1.12 Structural representation of R-roscovitine	51

### **CHAPTER 2**

2.1 Diagrammatic representation of the isolation of human granulocytes from whole blood	58
2.2 Photograph demonstrating the separation of freshly isolated human blood cells by Percoll <sup>TM</sup> density gradient	59
2.3 Flow cytometric dot plots of freshly isolated human granulocytes and monocytes demonstrating cell populations	60
2.4 Freshly isolated Diff-Quick <sup>TM</sup> stained granulocytes and mononuclear cells (x 1000 magnification)	61
2.5 Viable and apoptotic Diff-Quick <sup>TM</sup> stained human neutrophils 20 h (x 1000	62

magnification)

### **CHAPTER 3**

3.2.1 Effects of gliotoxin and TNF- $\alpha$ on neutrophil apoptosis at 2 h	77
3.2.2.1 Photomicrograph of freshly isolated neutrophils (x 1000 magnification)	79
3.2.2.2 Photomicrograph of the effect of R-roscovitine on neutrophil morphology at 4 h	80
3.2.3 Stimuli effecting neutrophil apoptosis at 20 h	82
3.2.4.1 Flow cytometric scatter plots showing unstimulated neutrophils and neutrophils stimulated with TNF- $\alpha$	84
3.2.4.2 Effect of R-roscovitine and gliotoxin on neutrophil activation assessed by shape change induced by GM-CSF, LPS, LTB <sub>4</sub> and TNF- $\alpha$	85
3.2.4.3 Effect of R-roscovitine and gliotoxin on neutrophil activation assessed by CD62L expression induced by GM-CSF, LPS, LTB <sub>4</sub> and TNF- $\alpha$	86
3.2.4.4 Effect of R-roscovitine and gliotoxin on neutrophil activation assessed by the expression of CD11b induced by GM-CSF, LPS, LTB <sub>4</sub> and TNF- $\alpha$	87
3.2.5 Effect of R-roscovitine and gliotoxin on macrophage phagocytosis of apoptotic neutrophils in the presence and absence of dexamethasone	91

### **CHAPTER 4**

4.1.1 Schematic representations of some of the signaling pathways involved in neutrophil survival and apoptosis	101
4.1.2 Simplified pictorial representation of the classical NF- $\kappa$ B pathway	103
4.1.3 Diagrammatic representation of the classical and alternative NF- $\kappa$ B pathways	105
4.2.1 Effect of inflammatory mediators on I $\kappa$ B $\alpha$ degradation in neutrophils	110
4.2.2 Effect of inflammatory mediators on I $\kappa$ B $\alpha$ degradation in neutrophils pre-treated with either R-roscovitine or gliotoxin	113
4.2.3 Effects of R-roscovitine and gliotoxin on LPS and TNF- $\alpha$ on movement of p65	115
4.2.4 Identification of the anti-apoptotic proteins cIAP, X-IAP and A1 in	117

neutrophil lysates	
4.2.5 Identification of Mcl-1 in neutrophils by Western Blotting and RT-PCR	119
4.2.6 Effect of R-roscovitine on GM-CSF induced neutrophil survival and the anti-apoptotic protein Mcl-1	121
4.2.7 Effect of R-roscovitine on GM-CSF, TNF- $\alpha$ and LPS induced neutrophil survival and the anti-apoptotic protein Mcl-1	124
4.2.8 Effect of proteasome and caspase inhibitors on R-roscovitine and GM-CSF mediation of Mcl-1 in neutrophils	125
4.2.9 Effect of R-roscovitine on the anti-apoptotic protein X-IAP	128
4.2.10.1 Diagrammatic representation of X-IAP-tat entering a neutrophil	130
4.2.10.2 Diagrammatic representation of the X-IAP-tat construct	131
4.2.10.3 Photomicrograph (x 400 magnification) of the effects of X-IAP-tat and R-roscovitine on neutrophil apoptosis at 18 h	132
4.2.10.4 Effects of the X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 18 h	133
4.2.11.1 Flow cytometric dot plots showing forward scatter versus side scatter and FL1 versus FL2 of neutrophils aged for 18 h with R-roscovitine	135
4.2.11.2 Flow Cytometric Dot plots showing forward scatter versus side scatter and FL1 versus FL2 of neutrophils aged for 18 h with either X-IAP-tat or X-IAP-tat + R-roscovitine	136
4.2.12 Effects of the X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 4 h in the presence or absence of LPS	139
4.2.13.1 Flow Cytometric Dot Plots showing forward scatter verses side scatter of neutrophils aged for 4 h with X-IAP-tat or R-roscovitine in the presence or absence of LPS.	140
4.2.13.2 Flow Cytometric histograms showing shape change induced in neutrophils aged for 4 h with X-IAP-tat or R-roscovitine in the presence or absence of LPS.	141
4.2.13.3 Histograms showing percentage shape change for neutrophils aged for 4 h with X-IAP-tat or R-roscovitine in the presence or absence of LPS.	142
4.2.14 Histograms showing collated data for neutrophil shape change and	143

CD62L expression induced by X-IAP-tat and LPS either in the presence or absence of autologous serum.

4.2.15 Standard curve calculated from serial dilutions of standards used to calculate the endotoxin concentration of the XIAP-tat construct. 146

## **Chapter 1**

### **Introduction**

#### **1.1 The inflammatory response**

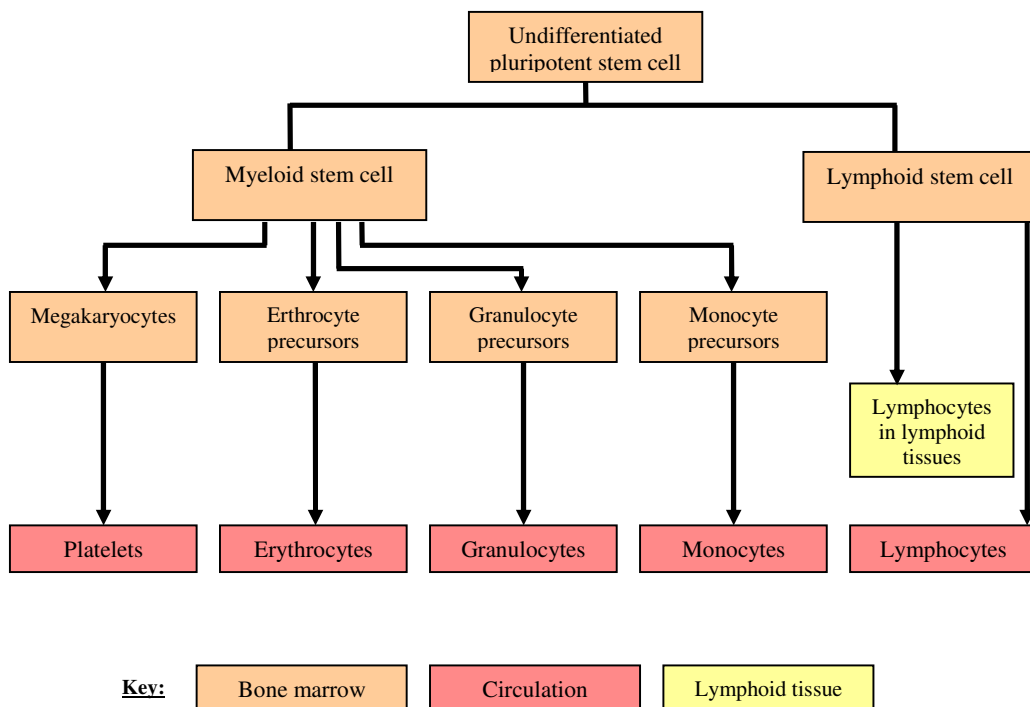
The inflammatory response is an innate highly complex, evolutionary conserved process of inter-related biochemical and cellular events which still remains shrouded in some mystery. When controlled effectively the inflammatory response defends the host organism against invasion by infectious agents, and/or tissue damage. This is achieved by detection, repression and abolition of the invading micro-organism before it can become established and compromise the host. The final occurrence essential to successful inflammatory responses is the resolution of inflammation, restoring tissue homeostasis (Leitch et al., 2009, Riley et al., 2006).

The inflammatory response was first defined by Celsus in approximately AD40 as “rubor, calor, dolor, tumor” also referred to as redness, heat, pain and swelling. These classical symptoms of inflammation are a result of localised vasodilation (in response to e.g. histamine production) increasing blood supply to the site of infection/injury and in turn the supply of phagocytic leucocytes, such as neutrophils, and plasma proteins, for example clotting factors. Histamine production is also responsible for increasing capillary permeability enabling movement of the plasma proteins to the site of inflammation. The net product of this is localised oedema due to an increase in interstitial fluid osmotic pressure. The pain associated with inflammation is a result of distension of the inflamed tissue and triggering of primary afferent neurons (Sherwood, 2001).

The main focus of this thesis is the human neutrophil and its role in the inflammatory response. Particular interest will be paid to neutrophil longevity as influenced by a variety of survival factors and the ability of cyclin-dependent kinase inhibitors to efficaciously induce neutrophil apoptosis. Therefore, the first section of this thesis will introduce the cells often referred to as the main cells involved in innate immune defence, the leucocytes.

## 1.2 Haematopoiesis

All circulating blood cells (granulocytes, monocytes, erythrocytes, megakaryocytes and lymphocytes) originate from the same pluripotent progenitor stem cell in the bone marrow (Figure 1.1). These pluripotent stem cells differentiate into the subsequent cell lineages according to the haematopoietic and growth factors they are exposed to. Haematopoietic and growth factors continue to influence and regulate blood cells at the inflammatory loci regulating their activation and inhibiting apoptosis (Danova and Aglietta, 1997).



**Figure 1.1 Haematopoiesis** – diagram adapted from Human Physiology Cells to Systems Forth Edition (Sherwood, 2001)

*All granulocytes, monocytes, erythrocytes, lymphocytes and platelets are derived from the same undifferentiated pluripotent stem cells. Exposure to Haematopoietic and growth factors influences the subsequent cell lineage formed. For example neutrophils are produced upon stimulation from cytokines such as G-CSF, GM-CSF, SCF, IL-3 and IL-6.*



### **1.3 Leucocytes**

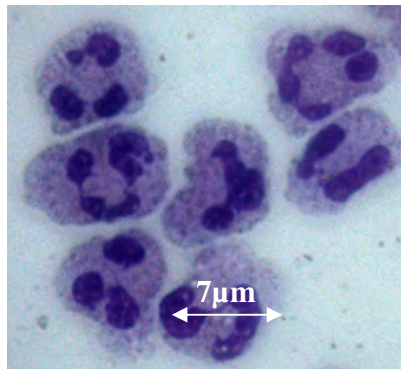
Leucocytes, also known as white blood cells, derive their name from their lack of haemoglobin which gives them a colourless appearance. The leucocytes can be subdivided into two distinct groups; the granulocytes (polymorphonuclear leucocytes) and the agranulocytes (mononuclear leucocytes; namely lymphocytes and monocytes). Leucocytes are derived in the bone marrow from a common precursor (Akashi et al., 2000) and this helps explain some of the common features between them.

The granulocytes; comprised of neutrophils, eosinophils and basophils obtain their name from the abundance of cytotoxic granules contained in their cytoplasm. They are the main cells involved in innate immune defence and are amongst the first of the cells to be recruited to the site of inflammation in copious numbers. Each type of granulocyte is predefined to specific stimuli (Duffin et al., 2010). Neutrophils are the most abundant of the circulating leucocytes in human blood, (approximately 50 – 70 %) defending the body against bacterial and fungal infections. Eosinophils, on the other hand, usually comprise only 1-4 % of the white blood cells in the circulation primarily defending the host against parasitic infection. Basophils are scarcer still (< 1 % of circulating leucocytes) and are thought to be involved in the resolution of parasitic infections and allergic disease (Riley et al., 2006).

### **1.4 The neutrophil**

The majority of the work conducted in this thesis will focus on the neutrophil. This is because neutrophils are the one of the most prevalent and rapidly recruited cell types of the innate immune system. They are easily identified by their multi-lobed nucleus (Figure 1.2). Neutrophils arise from CD34+ pluripotent stem cells located in the bone marrow under the stimulation of cytokines such as G-CSF, GM-CSF, SCF, IL-3 and IL-6. Marginated populations of mature neutrophils also reside in the microvasculature of various tissues such as the lungs; these in conjunction with increased production in the bone marrow are responsible for the rapid increase of circulatory neutrophils (approximately 10 fold) during

pro-inflammatory incidents. Throughout normal physiological conditions neutrophils are functionally dormant and have a very short half life of between 7 and 12 hours following which, if they have not infiltrated the tissues, it is proposed that they locate to the bone marrow, liver or spleen for disposal (Fox et al., 2010). However, upon functional activation neutrophils have a multifaceted role in protecting the host, not only eliminating invading pathogens through release of reactive oxygen species (ROS) and proteases, but also recruiting other immune cells and increasing the inflammatory response through the release of chemokines and cytokines (Cascao et al., 2009).



**Figure 1.2 Photomicrograph of freshly isolated human neutrophils**

*In the above picture the distinctive multi-lobed nucleus of the neutrophil can be seen. Neutrophils have an average diameter of approximately 7μm.*

### **1.5 Neutrophil recruitment to sites of inflammation**

In order for the neutrophils to carry out their defensive role it is essential that they are appropriately recruited to the site of infection. The process of neutrophil recruitment can be defined by the following four key stages: rolling; adhesion; diapedesis; and migration.

### 1.5.1 Rolling

Upon appropriate stimulation the process of rolling commences enabling transient interactions between the neutrophil and the endothelium using the selectins (E-selectin, L-selectin and P-selectin), a family of cell adhesion molecules which slow neutrophils at sites of inflammation (Choi et al., 2009, McEver, 2002). A prominent selectin involved in the process of rolling is L-selectin (also referred to as CD62L) which is constitutively expressed on the neutrophil surface membrane but its activity is potentiated upon neutrophil activation (I have measured the loss of expression of CD62L as a marker of neutrophil priming/activation later in this thesis). However, P-selectin and E-selectin are expressed on endothelial cell surface membranes (Choi et al., 2009). P-selectin is released from Weibel-Palade bodies upon exposure to either ROS, thrombin or histamine (Lowenstein et al., 2005). Whereas E-selectin is synthesized a couple of hours post release of the cytokines IL-1 and TNF- $\alpha$  from damaged cells, or by the presence of LPS from bacterial infection (Patel et al., 1995, Cascao et al., 2009). All three selectin glycoproteins are recognised by the P-selectin glycoprotein ligand (PSGL-1) expressed on both endothelial and neutrophil cell surface membranes. The interaction between P-selectin and PSGL-1 is thought to initiate the initial tethering of the neutrophil to the endothelium and E-selectin, which is also recognised by E-selectin ligand 1 (ESL-1), is thought to be involved in more secure rolling (Choi et al., 2009). Rolling enables neutrophils to detect inflammatory chemokines such as IL-8 and chemoattractants for example LTB<sub>4</sub>, PAF and C5a necessary for priming the neutrophil for adhesion (Cascao et al., 2009, Choi et al., 2009).

### 1.5.2 Adhesion

The next stage of the neutrophil's journey is firm and stationary adhesion to the endothelium, mediated by the neutrophil  $\beta_2$ -integrins. The  $\beta_2$ -integrins are heterodimers composed of an  $\alpha$  subunit (either CD11a, CD11b or CD11c) and a  $\beta$  subunit CD18. The most prevalent  $\beta_2$ -integrins are  $\alpha$ L $\beta$ 2 (LFA-1) comprised of CD11a/CD18 and  $\alpha$ M $\beta$ 2 (Mac-1) comprised of CD11b/CD18, which interact with the endothelial cell surface membrane by either, ICAM-1, ICAM-2 ( $\alpha$ L $\beta$ 2 and  $\alpha$ M $\beta$ 2), or the receptor for advanced glycation end

products (RAGE) ( $\alpha$ M $\beta$ 2) (Yonekawa and Harlan, 2005). Chemokines activate integrin expression on neutrophils by several pathways such as small GTPases, and adhesion of the neutrophil to the endothelium is increased once integrin-ligand binding has occurred through conformational changes and clustering of the integrins.  $\alpha$ L $\beta$ 2 and  $\alpha$ M $\beta$ 2 integrin bindings are enhanced through Src-like protein tyrosine kinases. Neutrophils move by locomotion mediated by  $\beta$ <sub>2</sub>-integrin binding until they reach the closest junction between endothelial cells (Yonekawa and Harlan, 2005, Choi et al., 2009, Cascao et al., 2009).

### 1.5.3 Diapedesis

To begin its journey through the endothelium the neutrophil must first overcome VE-cadherin, the full process of which has not been elucidated and it is not clear if the neutrophil's interaction with and the subsequent vanishing of VE-cadherin is essential for, or a product of, migration (Choi et al., 2009). Neutrophil diapedesis is primarily mediated by the Ig-superfamily of adhesion molecules, namely platelet endothelial cell adhesion molecule-1 (PECAM-1) and junctional adhesion molecules (JAMs) the former being expressed by neutrophils and by endothelial cells at junctions whilst the latter is solely expressed on endothelial cells at tight junctions. PECAM-1 enables transmigration through homophilic interactions, which also act to increase levels of the laminin receptor  $\alpha$ 6 $\beta$ 1-integrin on neutrophils which aids the neutrophils ability to transverse the basement membrane of the endothelium. Interestingly it has been discovered (in mice) that CD99L2, expressed on both neutrophils and at endothelial junctions is essential to diapedesis, inhibition of which prevents the neutrophils ability to pass through the perivascular membrane (Bixel et al., 2004). The mode of action of JAMs is also homophilic; however, they can also interact with  $\beta$ <sub>2</sub>-integrins, JAM-A binding with  $\alpha$ L $\beta$ 2 and JAM-C with  $\alpha$ M $\beta$ 2. ICAM-1 also has a proposed involvement in the migration of neutrophils through the endothelium. Neutrophil movement through the epithelium is triggered by the chemotactic secretion of IL-8 from epithelial cells which have experienced an inflammatory insult and from bacterially secreted formyl peptides (Cascao et al., 2009, Choi et al., 2009).

#### **1.5.4 Migration**

The final step in the process is mobilisation of the neutrophil to the inflamed tissue along chemotactic gradients. Various cells can release different chemoattractants, for example PAF is released by endothelial cells, granulocytes, platelets and macrophages. LTB<sub>4</sub> is produced by monocytes and granulocytes. C5a is produced in the blood during the classical complement cascade, invading bacteria release chemoattractive peptides such as formylated peptides (fMLP) and stimulate the release of cellular chemokines, a family of approximately 40 proteins such as IL-8 produced by a wide range of cells including endothelial, epithelial, macrophages, monocytes, platelets, neutrophils and parenchymal cells (Wagner and Roth, 2000). It has been proposed that due to the extensive, redundant actions of different chemoattractants, crosstalk may occur between receptors and active intracellular pathways prioritising one attractant over another and helping guide the neutrophil to the area of tissue requiring the most attention. Chemoattractants mainly mediate migration through  $\beta_1$ ,  $\beta_2$  and  $\beta_3$ -integrins, enabling the leading edge of the neutrophil to adhere, with the help of anti-adhesive molecules such as CD34 detaching the rear of the cell (Cascao et al., 2009).

#### **1.6 Neutrophil granules and secretory vesicles**

The neutrophil's cytoplasm contains an abundance of cytotoxic granules. These house an arsenal of more than 300 proteins which are deployed upon activation of the neutrophil and are involved in vital processes such as adhesion, migration and protecting the host organism from invading pathogens (Borregaard and Cowland, 1997). When neutrophils come into contact with invading organisms they exploit these granules in one of three ways; they either phagocytose the pathogen, releasing into the phagolysosome reactive oxygen species and the products of the granules; or they exocytose the granules contents towards the organism. In 2007 a third method was discovered by which activated neutrophils can defend against foreign bodies, this is the deployment of extracellular traps (Fuchs et al., 2007, Brinkmann and Zychlinsky, 2007). These traps are comprised of chromatin along with granule constituent fibers and capture and kill the target organism. This process,

however, is ultimately fatal for the neutrophil as it is accompanied by cell membrane rupture.

Neutrophil granules can be subdivided into the following types: azurophil granules which are also referred to as primary granules as they are the first of the three distinct types of granule to be formed during myeloid cell differentiation or “peroxidase-positive granules” due to their high concentrations of myeloperoxidase. These can be further classified into defensin-poor azurophil granules which precede the later developing classification of defensin-rich azurophil granules (Arnljots et al., 1998). Interestingly the azurophil granules predominantly function is within the phagolysosome, helping to destroy the engulfed pathogen (Joiner et al., 1989) and as a result are not primarily involved in exocytosis (Faurischou and Borregaard, 2003). Azurophilic granules are the last of the three types of granules to be mobilised as they contain a high percentage of the neutrophil’s anti-microbial arsenal.

The granules derived, post promyelocyte/myelocyte transition, are formed once the production of myeloperoxidase has terminated and therefore are referred to as peroxidase-negative granules. Peroxidase-negative granules can be sub-classified into specific granules (also called secondary granules as they are the second type of granule formed during neutrophil formation) and gelatinase granules (named after their high concentration of gelatinase and also known as tertiary granules as they are the third of the granules to appear) (Kjeldsen et al., 1992).

Neutrophils also contain a forth exocytic mechanism called secretory vesicles. These form a distinct category from the granules as they contain plasma proteins, identifying them as endocytic in origin. They are highly responsive to stimulation, this helps explain the ability of the neutrophil to upregulate  $\beta$ -2 integrin by chemoattractants which do not stimulate movement of the granules to the cell membrane (Hager et al., 2010). The secretory vesicles are implicated in neutrophil recruitment to inflammatory loci and thus are the first of the granule family to migrate to the cell surface membrane upon stimulation by an extensive list of inflammatory stimuli (Fox et al., 2010). The most prominent constituents of the

secretory vesicles are complement receptor 1 (CR1), CD11b, CD14, CD16 the Fc $\gamma$ III receptor, receptors for formylated bacterial proteins (fMLP-receptors) and leukolysin a metalloprotease. In conjunction with the mobilisation of the secretory vesicles to the cell surface membrane, CD62L (L-selectin) is shed from the surface membrane. This enables the neutrophil to initiate firm binding to the activated endothelium at sites of inflammation initiating the process of transmigration (Faurschou and Borregaard, 2003). Interestingly it has been reported that mobilisation of the secretory vesicles is implicated in increased concentrations of the proposed monocytes chemoattractant heparin-binding protein (HBP) thus attracting monocytes to the inflamed area which will help aid resolution of inflammation (Soehnlein et al., 2005).

<b><u>Neutrophil Granules</u></b>			
<b><u>Azurophil/ Primary Granules</u></b>	<b><u>Specific/ Secondary Granules</u></b>	<b><u>Gelatinase/ Tertiary Granules</u></b>	<b><u>Secretory Vesicles</u></b>
Myeloperoxidase	Lactoferrin	Gelatinase	CD11b
Lysozyme	Lysozyme	Plasminogen Activator	CR1
Defensins	Collagenase	Cathepsin B	CD14
Bacterial/Permeability-Increasing Protein	Complement Activator	Cathepsin D	CD16
Elastase	Phospholipase A <sub>2</sub>	β-D-Glucuronidase	Leukolysin
Cathepsin G	CR3	α-Mannosidase	fMLP-receptors
Proteinase 3	CR4	Cytochrome b <sub>558</sub>	Heparin-binding protein
Cathepsin B	FMLP Receptors	CD11b	
Cathepsin D	Laminin Receptors		
β-D-Glucuronidase	CD11b		
α-Mannosidase	Cytochrome b <sub>558</sub>		
Phospholipase A <sub>2</sub>	Monocyte-Chemotactic Factor		
Chondroitin-4-Sulphate	Histamine		
	Vitamin B <sub>12</sub> Binding Protein		

**Figure 1.3 Table showing the main constituents of neutrophil granules and secretory vesicles**

*The table above shows the 3 types of granules and secretory vesicles housed within the neutrophil. Listed are some of the key factors they contain which are implicated in functions such as adhesion, migration and protecting the host organism from invading pathogens.*

### **1.7 Monocytes**

Another cell pivotally involved in the inflammatory response is the monocyte. Monocytes are produced by the same pluripotential stem cells in the bone marrow that produce the granulocytes, erythrocytes, lymphocytes and platelets. Monocytes play an important role in the body's defence system as they are the precursor cells of phagocytic macrophages (Latin



for big eater) and dendritic cells, replenishing populations in the peripheral tissues. Furthermore, they also have a direct immune function defending against invading pathogens. Monocytes arrive at the site of inflammation later than the neutrophils, approximately 8-12 hours post host insult.

There are two distinct sub-classifications of Monocytes defined by their expression of CD14 (induced in LPS recognition) and CD16 (Fc $\gamma$ RIII immunoglobulin receptor) which exhibit discrete functional and phenotypical features. These are the classical human CD14<sub>high</sub> CD16<sup>-</sup> which comprise 80-90% of monocytes in the circulation (Serbina et al., 2008) and are implicated in the initiation of inflammation, giving rise to macrophages and dendritic cells involved in pathogen clearance, resolution of inflammation and antigen presentation (Gordon, 2007). The second group are the non classical human CD14<sub>low</sub> CD16<sup>+</sup>, accounting for approximately 10 % of the circulating monocytes under normal homeostatic conditions (Serbina et al., 2008) which form resident tissue dendritic cells and macrophages such as splenic (Gordon, 2007). However, the exact roles of the two sub-classifications of monocytes have yet to be fully elucidated, as has their precise functions once they have entered the tissues (Soehnlein and Lindbom, 2010).

## **1.8 Macrophage**

Macrophages are key effector cells in both the innate and adaptive immune system contributing to the former by phagocytosising invading organisms and cellular debris such as apoptotic neutrophils, and to the latter by antigen presentation.

It has been widely reported that at least two distinct phenotypically different types of polarised macrophages exist, determined by the environment surrounding them. These are M1 classically activated macrophages that are pro-inflammatory in nature, and M2 alternatively activated macrophages which are considered to be anti-inflammatory. M1 macrophages are produced upon stimulation by type 1 inflammatory cytokines (produced by Type 1 T helper cells) and products from invading organisms. However, M2 macrophages are the product of a Type 2 T helper cell rich environment and more recently

have been reclassified to M2a (induced by IL-4 and IL-13), M2b (induced by agonists of IL-1 receptors, TLRs and immune complexes) and M2c (induced by glucocorticoids and IL-10) subtypes, defined by the different activation markers they express (Benoit et al., 2008, Mantovani et al., 2004). Furthermore, another recent discovery of an additional phenotype has been identified in the form of the apoptotic cell phagocytosing resolution phase macrophage (Bystrom et al., 2008). This has led to the proposal that macrophages should not be defined as a set phenotype but instead as polymorphic, able to alter their phenotype depending upon their environment, allowing them to participate in all stages of inflammation (pro-inflammatory, anti-inflammatory and resolution) (Porcheray et al., 2005, Stout and Suttles, 2004, Benoit et al., 2008). The macrophage and its role in the resolution of inflammation will be discussed in greater detail later in the chapter.

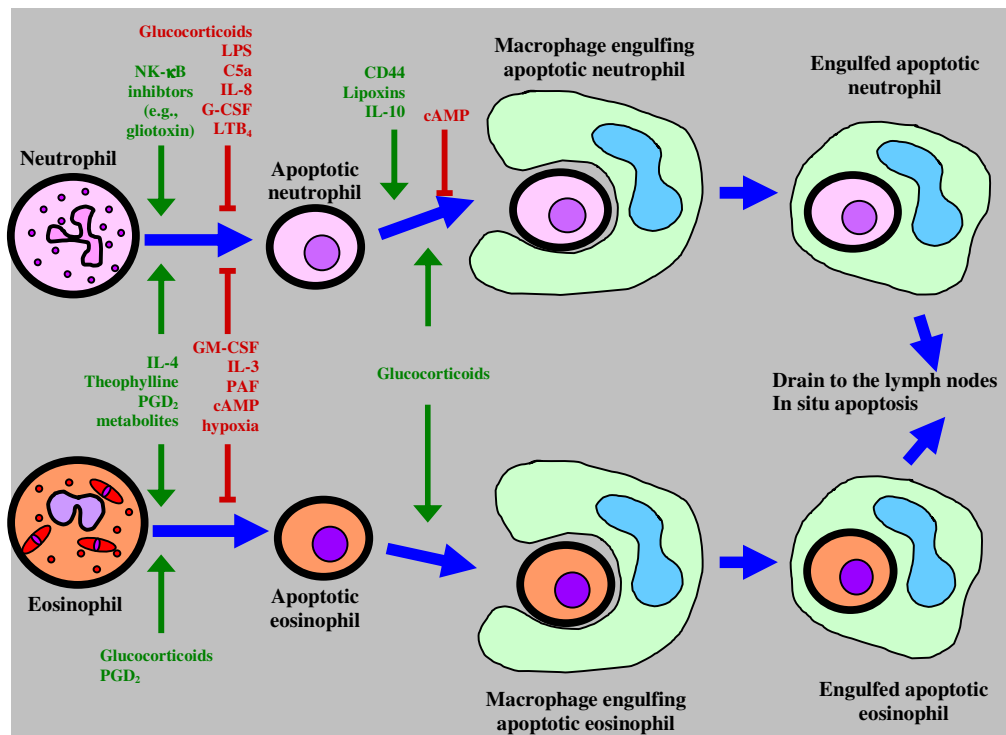
## **1.9 Apoptosis**

### **1.9.1 Structural and cellular changes occurring during granulocyte apoptosis**

Apoptosis is an evolutionary conserved pathway and like virtually all cells, granulocytes undergo this form of programmed cell death (Wyllie et al., 1980, Savill et al., 1989). Granulocytes, having left the bone marrow, enter the circulation (it is estimated that the half life of neutrophils in the circulation is approximately 6 hours) and in response to appropriate stimuli enter the tissues. Once their physiological role (e.g., killing and digestion of invading organisms) has been accomplished the cells are destined to undergo spontaneous or constitutive apoptosis in the tissues. Isolated human neutrophils under optimal *in vitro* culture conditions can survive up to 20-40 hours whereas eosinophils *in vitro* tend to survive longer (40-80 hours) before apoptosis is engaged. Apoptosis can be delayed significantly by a number of environmental factors and mediators. However, once the process of apoptosis has been initiated (usually by the activation of caspases – see below) there is a reduction in granulocyte volume, their chromatin condenses and their DNA is cleaved internucleosomally. Most cell types fragment, resulting in the release of many membrane encapsulated apoptotic bodies. However, granulocytes tend to stay intact resulting in the formation of relatively few apoptotic bodies per cell. The apoptotic bodies are membrane enclosed thereby preventing release of the intracellular contents into the

extracellular milieu, and preventing damage to neighbouring cells and tissues. Apoptotic cells are then rapidly recognised and engulfed by phagocytes (e.g., macrophages) in response to the exposure of phagocytosis signals (e.g., phosphatidylserine (PS)) on the surface of the apoptotic cell (Savill et al., 2002, Gregory and Devitt, 2004).

Manipulation of this process therefore poses huge potential for the treatment of inflammatory disorders. Therefore in order for this to occur it is essential that every aspect of apoptosis from cell type specific variations to general regulatory mechanisms are fully understood.



**Figure 1.4 Factors influencing human granulocyte apoptosis.**

*The half-life of granulocytes is regulated at sites of inflammation by numerous mediators which can either increase or decrease rates of apoptosis. Some of these, for example, the haemopoietic and inflammatory agents GM-CSF, IL-3, PAF, cAMP and a hypoxic environment can inhibit both neutrophil and eosinophil apoptosis, whereas agents such as glucocorticoids can enhance eosinophil apoptosis but conversely inhibit neutrophil apoptosis. They can also act to modulate the rate at which apoptotic cells are recognised and phagocytosed by professional phagocytes. Glucocorticoids, for example, augment the phagocytic removal of apoptotic cells. (Riley et al., 2006)*

### 1.9.2 Apoptotic pathways

There are a number of signaling pathways that can regulate the rate of apoptosis in granulocytes, like most other cells, apoptosis can be initiated *via* two conserved pathways, commonly known as the extrinsic or the intrinsic pathways.

#### 1.9.2.1 Extrinsic Pathway

The extrinsic pathway brings about apoptosis through the stimulation of cell surface ‘death receptors’ for example TNFR, Fas, and TRAILR (all of which are present on human neutrophils (Akgul and Edwards, 2003), and eosinophils (Daigle and Simon, 2001) by specific counter ligands. Interestingly, the ligands for these receptors not only initiate apoptotic pathways but can also impact upon other signaling pathways that influence cellular responsiveness. For example, the role of the TNF- $\alpha$  receptors in neutrophils is particularly notable as they have the capacity to ‘prime’ the cells for subsequent enhanced stimulation by other agonists (e.g., formylated peptides) and infer both pro-apoptotic and anti-apoptotic signaling depending upon the length of the incubation period with TNF- $\alpha$ . Culture of neutrophils with TNF- $\alpha$  for 12 h or longer have been reported to delay apoptosis, whilst conversely periods of culture of 8 h or less appear to trigger apoptosis in sub-populations of neutrophils (Murray et al., 1997). The pro-apoptotic and anti-apoptotic pathways are activated upon binding of TNF- $\alpha$  to TNFR1 (TNF receptor 1) and TNFR2 (Murray et al., 1997), (Walmsley et al., 2004). The receptors trimerise inducing proximity of their death domains, consequently allowing the death domains of TNFR-associated death domain-containing proteins (TRADDs) to bind. TRADD can also induce apoptosis through binding to FADD subsequently activating pro-caspase-8 and has the ability to activate the transcription factors NF- $\kappa$ B and AP-1 via binding to secondary adaptor molecules such as TNFR-associated factor-2 and Receptor-Interacting Protein (RIP). This may implicate TNF- $\alpha$  as also having a role in protecting the cell from apoptosis as NF- $\kappa$ B is known to induce anti-apoptotic factors.

### **1.9.2.2 Intrinsic Pathway**

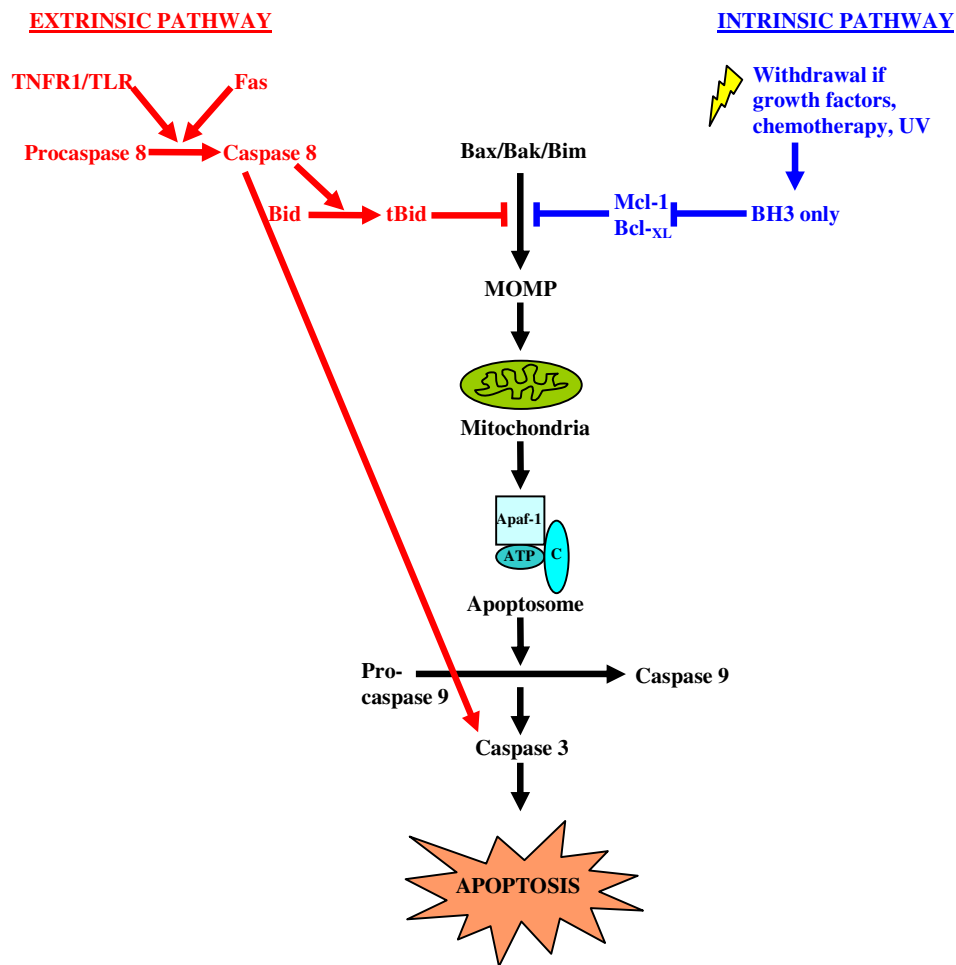
Cellular stress or injury triggered by, for example, UV irradiation or withdrawal of growth or survival factors activates the intrinsic apoptotic pathway. In this instance the key players in the initiation of apoptosis are a family of proteins called the Bcl-2 family, which control the release of cytochrome C and other apoptogenic from the mitochondria by increasing outer membrane permeability.

### **1.9.3 Cysteine-dependent aspartate-specific protease family (Caspases)**

As has been mentioned above much of the initiation and execution of apoptosis is mediated via the caspase family of enzymes. The caspases are a family of death promoting proteolytic enzymes that become activated through the cleavage of their precursor molecule to generate an active caspase during apoptosis. They derive their name from ubiquitously contained cysteine residues within their active sites, which upon activation of the enzyme, cleave aspartic acid residues in their target proteins eliciting a cascade of destruction that ultimately brings about the demise of the cell. Caspases involved in apoptosis can be divided into two classes: initiator caspases and effector caspases. Initiator caspases (caspases 8, 9 and 10) as their name suggests bring about the initiation of the extrinsic (e.g., death receptor) apoptotic pathway. Procaspsases 8 and 10 do this *via* binding to adaptor molecules, for example, Fas-associated death domain (FADD) using their long death effector domain (DED) containing prodomains. In addition initiator caspases can be activated by autocatalysis, which is also dependent on their prodomains, as in this instance they act to bring about the oligomerization of the initiator pro-caspases aiding autoactivation. Consequently, initiator caspases can proceed to activate the downstream effector caspases (caspases 3, 6 and 7) *via* cleavage of the effector procaspases resulting in morphological changes. Neutrophils and eosinophils have been shown to possess both initiator and effector caspases (Riedl and Shi, 2004, Creagh et al., 2003).

#### **1.9.4 Cross Talk between Intrinsic and Extrinsic pathways**

Although in most circumstances the two pathways described above occur independently, they can be integrated by the pro-apoptotic Bcl-2 family member Bid. The crosstalk phenomenon is initiated via caspase 8 cleaving Bid to form truncated Bid (tBid) which then translocates to the mitochondria increasing the permeability of its outer membrane resulting in the release of cytochrome C. Cytochrome C together with Apaf-1 forms the apoptosome resulting in induced proximity of procaspase-9 molecules. This results in activation of caspase-9, which in turn activates procaspase-3 resulting in the cleavage of substrate proteins, inducing the morphological changes associated with apoptosis. Cells in which cross talk occurs are referred to as type II cells (in type I cells caspase-8 directly cleaves pro-caspase 3) and it has been proposed that neutrophils fall within the type II classification (Riley et al., 2006).



**Figure 1.5 Diagrammatic representations of the extrinsic and intrinsic apoptotic pathways and cross talk between the two. Adapted from (Mandelin and Pope, 2007)**

*In certain circumstances cross-talk can occur between the intrinsic and extrinsic apoptotic pathways. This occurs when Bid is cleaved by caspase 8 to produce tBid, which can increase permeability of the mitochondrial outer membrane allowing the release of Cytochrome C. This enables the formation of the apoptosome and subsequently activation of caspase 9 and caspase 3 resulting of the cleavage of substrate proteins inducing apoptosis.*



### 1.10 Neutrophil Survival Proteins

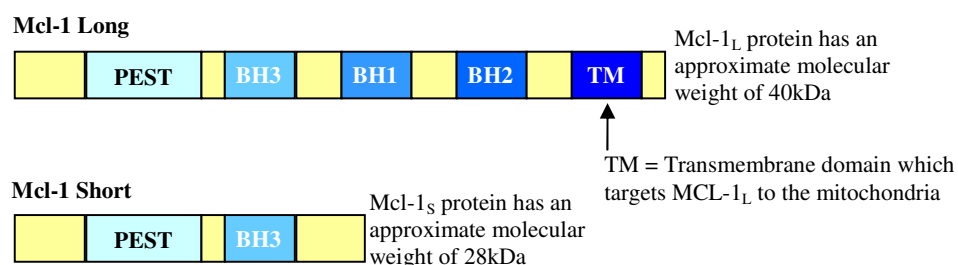
One aspect of this study will be the role of anti-apoptotic proteins in regulating human granulocyte survival, of which some of the most infamous belong to the Bcl-2 family. Interactions between the Bcl-2 family members play an essential role in deciding the fate of a cell. Members of the Bcl-2 family are a series of cell death regulatory proteins, of which there have been over 20 members identified so far (Baliga and Kumar, 2002). They are divided into three subgroups depending upon their function and the number of Bcl-2 Homology (BH) domains they contain, of which there are four in total. These subgroups are the anti-apoptotic Bcl-2 family and the pro-apoptotic family. The pro-apoptotic family can be further sub classified into multi-domain pro-apoptotic Bcl-2 members Bax and Bak, which are essential for the downstream permeabilisation of the mitochondria and activation of the caspases, and BH3 domain only pro-apoptotic Bcl-2 members which initiate apoptosis through binding to their Bcl-2 anti-apoptotic partners. Neutrophils and eosinophils are known to contain various members of the Bcl-2 family which play an essential role in regulating their apoptotic processes (Riley et al., 2006).

Neutrophils whilst lacking the anti-apoptotic protein Bcl-2 are known to express the Bcl-2 homologue A1 (also referred to as BFL-1) and the anti-apoptotic proteins Mcl-1, Bcl-X<sub>L</sub> and X-IAP a member of the Inhibitor of Apoptosis (IAP) family. The best studied of these proteins in neutrophils is Mcl-1. Mcl-1 differs from the other anti-apoptotic multi-domain members of the Bcl-2 family (A1, Bcl-2 and Bcl-X<sub>L</sub>) which contain four BH domains whereas Mcl-1 only possesses three. It has been widely reported that Mcl-1 protein levels in blood circulating neutrophils decrease preceding the cell entering apoptosis. Inflammatory mediators that postpone neutrophil apoptosis do this (at least in part) through maintaining or increasing levels of Mcl-1 and/or A1 enabling neutrophils to carry out their vital defensive functions. The half lives of both A1 and Mcl-1 mRNA along with the Mcl-1 protein are known to be short in contrast to the long half lives of the pro-apoptotic Bcl-2 members. This offers an explanation as to why neutrophils are 'primed' for apoptosis when in the circulation as *de novo* synthesis of Mcl-1 and A1 is absent, therefore allowing the effect of long lived proapoptotic proteins to pre-dominate (Moulding et al., 1998, Derouet

et al., 2004, Edwards et al., 2004). Survival signals in the form of cytokines and other mediators found at sites of inflammation induce *de novo* synthesis of the anti-apoptotic proteins, demonstrated through the use of agents that inhibit protein synthesis (e.g. cycloheximide and actinomycin D) which prevent increased granulocyte survival and directly induced apoptosis (Whyte et al., 1997).

### 1.10.1 Myeloid cell leukaemia differentiation protein (Mcl-1)

As Mcl-1 has been demonstrated to play a significant role in neutrophil apoptosis particular attention has been played to the intricate involvement of this protein. Mcl-1 derived its name as it was originally isolated from a human myeloblastic leukaemia cell line. In 2007 Dzhagalov *et al.* discovered through the use of conditional Mcl-1 knock out mice that Mcl-1 is an essential non redundant survival protein in neutrophils demonstrated by severely impaired neutrophil survival in the knock out mice. However, this does not appear to be the case with macrophages as the knock out mice possessed a normally functioning macrophage population (Dzhagalov et al., 2007).



**Figure 1.6 Diagrammatic representations of the two isoforms of Mcl-1**

*Mcl-1 Long* is the anti-apoptotic, wild type, full length protein encoded from all 3 exons of the *Mcl-1* gene. It contains BH domains 1, 2 and 3, a transmembrane domain which targets the protein to the mitochondria, and a PEST sequence associated with rapid turnover of the protein. *Mcl-1 Short*, however, is encoded from just 2 exons of the *Mcl-1* gene (the second exon is skipped) and as a result lacks BH1, BH2 and the transmembrane domain, and instead behaves like a member of the pro-apoptotic BH3 domain only family, such as *Bid*.

Mcl-1 is a particularly interesting member of the Bcl-2 family as it can take the form of one of two splice variants referred to as Mcl-1 long (Mcl-1<sub>L</sub>) and Mcl-1 short (Mcl-1<sub>S</sub>). Mcl-1<sub>L</sub> is the wild type full length protein encoded from all 3 exons which exhibits its infamous role as an anti-apoptotic protein. However, Mcl-1<sub>S</sub> skips the second exon resulting in the translated protein retaining the BH3 domain but losing the BH2, BH1 and transmembrane domains. Interestingly, as the Mcl-1<sub>S</sub> protein contains the BH3 domain it therefore possesses properties similar to the BH3 only domain family of pro-apoptotic proteins such as Bid, Bad and Bim, and when over-expressed has the ability to induce apoptosis (Bingle et al., 2000). Furthermore, Mcl-1<sub>S</sub> has been demonstrated to form dimers with Mcl-1<sub>L</sub> enabling the anti-apoptotic ability of Mcl-1<sub>L</sub> to oppose the pro-apoptotic action of Mcl-1<sub>S</sub>. This implies that the fate of the neutrophil could be determined by the dominant isoform of Mcl-1 expressed in the cells (Bae et al., 2000). This thesis will focus on the wild type Mcl-1<sub>L</sub> which from here on will be referred to as Mcl-1 unless otherwise indicated.

Mcl-1 can also be regulated at the transcriptional level and interestingly it has been proposed that short nucleotide polymorphisms (6 and 18 nucleotides long) in the promoter region of the Mcl-1 gene are associated with increased expression of Mcl-1 mRNA and protein in haemopoietic and epithelial cells (Saxena et al., 2007). These short nucleotide polymorphisms have been found in individuals with Chronic Lymphocytic Leukaemia (CLL) and healthy individuals, although the prevalence varies between studies from 25 – 60 % of CLL patients to 4 – 70 % of varying healthy populations (Saxena et al., 2007).

Another feature making Mcl-1 stand apart from its fellow Bcl-2 anti-apoptotic family members is that it contains a PEST domain ((P) proline, (E) glutamic acid, (S) serine, (T) threonine rich domain) which is associated with proteins that have a short half life (Akgul et al., 2000b). However, Akgul *et al.* made the interesting observation that deletion of this domain did not affect the stability of the Mcl-1 protein, indicating that the PEST motif is not solely responsible for the rapid turn over of the Mcl-1 protein.

Other proposed methods of regulation are by proteolytic degradation by the caspases (Herrant et al., 2004) and granzyme B (Han et al., 2004), or proteasomal degradation

(Derouet et al., 2004). MULE a ubiquitin E3 ligase which possesses a BH3 domain therefore enabling Mcl-1 directed action has been identified (Zhong et al., 2005). However, another layer of complexity was added to the story when it was reported in 2010 that Mcl-1 regulation is not impeded by the deletion of the sites for ubiquitination and that the turn-over of the mutated protein resembles that of the wild-type in both normal and apoptosis inducing conditions. Furthermore, Mcl-1 could also be degraded by the 20S proteasome in an un-ubiquitinated state (Stewart et al., 2010).

Thus to summarise the Mcl-1 protein is subject to multiple redundant methods of steady state protein regulation, which perhaps is not surprising for a protein so intricately involved in the ultimate fate of the cell.

### **1.10.2 Mcl-1 function**

Like the majority of Bcl-2 family members wild type Mcl-1 is targeted to the mitochondrial membrane by the hydrophobic transmembrane domain located at the C-terminus of the protein (Akgul et al., 2004). Mcl-1 exerts its pro-survival function via interacting with numerous pro-apoptotic proteins.

One major function of Mcl-1 in viable cells is to bind to the multidomain pro-apoptotic protein Bak (located on the mitochondrial membrane) and hold it in an inactive conformation. Upon initiation of apoptosis, for example by DNA damage, Noxa (a BH3-only proapoptotic protein) is activated, interaction between Noxa and Mcl-1 results in displacement of Bak marking Mcl-1 for degradation by the proteasome. This enables Bak to undergo conformational changes and homo-oligomerization triggering permeabilisation of the mitochondrial membrane and allowing release of cytochrome C and other apoptogenic factors. It should be noted that Bcl-X<sub>L</sub> can also suppress Bak and that in order for apoptosis to be induced Bak must not be bound to either Bcl-X<sub>L</sub> or Mcl-1 (Willis et al., 2005).

It has also been speculated that Mcl-1 plays a role in the inhibition of Bax, another multidomain pro-apoptotic protein responsible for permeabilisation of the mitochondrial membrane. Unlike Bak, in viable cells Bax mainly resides in the cytoplasm. However, upon activation Bax translocates to the mitochondrial membrane and it is here that Mcl-1 is proposed to perform its inhibitory function. Interestingly the inhibitory action of Mcl-1 on Bax has yet to be fully elucidated and is independent of an interaction between Mcl-1 and Bax. Yet in order for Mcl-1 to exert its pro-survival function Bax needs to be present (Germain et al., 2008).

As previously mentioned BH3 only pro-apoptotic members function upstream of Bax and Bak and can not induce apoptosis in their absence. One such member of the BH3 only proteins is Bim. Bim is a promiscuous member of the BH3 only family and can bind to all anti-apoptotic members, including Mcl-1 (Adams and Cory, 2007). In viable cells Mcl-1 binds Bim on the outer mitochondrial membrane and neutralises its proapoptotic ability. However, upon initiation of extrinsic TRAIL-mediated apoptotic pathway activation of caspase 8 and caspase 3 degrades Mcl-1 relieving its inhibition on Bim. This then enables Bim to elicit Bax-dependent apoptosis highlighting a possible second mechanism of cross talk between the extrinsic and intrinsic apoptotic pathways (Han et al., 2006). Interestingly, Mcl-1 is also involved in classical cross talk between the extrinsic and intrinsic pathways (as described in section 1.9.4) as Mcl-1 binds potently to tBid inhibiting mitochondrial outer membrane permeabilisation (MOMP) (Clohessy et al., 2006).

### **1.10.3 Role of Mcl-1 in the neutrophil**

As mentioned above survival signals and mediators located at inflammatory loci can stimulate production of survival proteins. One such cytokine known to increase neutrophil survival that is found elevated levels at sites of inflammation is granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is known to increase the levels of Mcl-1 protein through stimulating transcription of the Mcl-1 gene (Derouet et al., 2004). Proposed mechanisms for the transcriptional upregulation of Mcl-1 are through MAPK influencing SRF/Elk-1 (Townsend et al., 1999) (Townsend et al., 1998), or alternatively via the

PI3K/Akt CREB transcription factor pathway (Wang et al., 1999). For more information of MAPK and PI3K pathways and their roles in granulocyte apoptosis please see 1.11.2 PI3K Pathway and 1.11.3 MAPK Pathway.

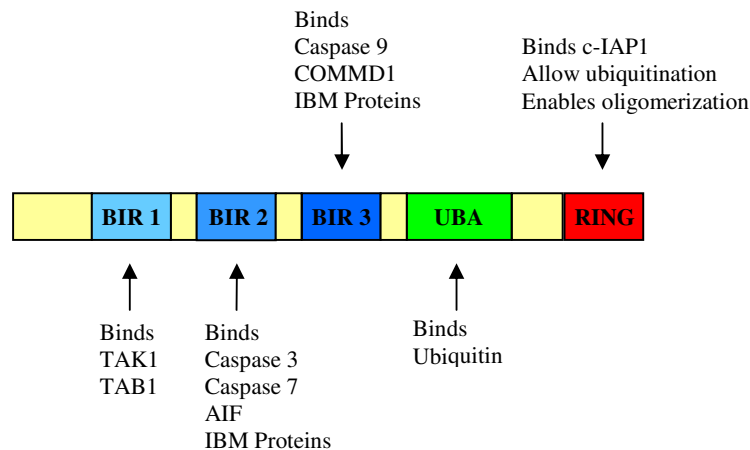
However this may not be the sole way through which GM-CSF influences Mcl-1 levels. Interestingly it has also been shown that GM-CSF increases the cellular concentration of Mcl-1 in neutrophils to a more significant level than those achieved through enhanced de novo synthesis of the Mcl-1 protein by increasing the stability of the Mcl-1 protein and reducing the rate with which it is turned over via the proteasome (Derouet et al., 2004).

As mentioned in 1.9.2 Apoptotic pathways – extrinsic pathway, TNF- $\alpha$  exposure can have converse effects on neutrophil apoptosis depending upon the length of exposure (Murray et al., 1997, Walmsley et al., 2004), and the concentration neutrophils are exposed to with low concentrations of TNF- $\alpha$  having a protective effect whilst high concentrations have a pro-apoptotic effect on neutrophils (van den Berg et al., 2001). Cross proposed in 2008 that the differing concentration dependent actions of TNF- $\alpha$  result from differing pathways being triggered in the neutrophil, one of which involves Mcl-1. At low concentrations of TNF- $\alpha$  (defined as <1ng/ml) induced neutrophil production of the anti-apoptotic protein A1 and subsequent neutrophil survival. However at high concentrations (>10ng/ml) TNF- $\alpha$  stimulated caspase-dependent decrease in Mcl-1 levels priming the cells for apoptosis (Cross et al., 2008). Interestingly unlike GM-CSF, TNF- $\alpha$  does not induce transcription of Mcl-1 or accelerate turnover of Mcl-1 by the proteasome.

Interestingly Mcl-1 regulation also plays an important role in the ability of the commonly prescribed pharmacological agent aspirin to promote resolution of inflammation. Aspirin triggers 15-epi-lipoxin A4 (15-epi-LXA4)) and has been demonstrated to decrease levels of Mcl-1 in human neutrophils. This is achieved through 15-epi-LXA4 preventing the ability of myeloperoxidase (MPO) to induce an increase of Mcl-1 promoting apoptosis and the resolution of inflammation (El Kebir et al., 2009).

#### 1.10.4 X-Linked Inhibitor of Apoptosis (X-IAP)

Another anti-apoptotic protein of particular interest in this thesis is X-IAP, a member of the inhibitor of apoptosis protein family (IAPs) of which to date eight have been identified in humans. Members of the IAP family are defined by the possession of one – three baculovirus IAP repeat (BIR) zinc-binding motifs (Zangemeister-Wittke and Simon, 2004).



**Figure 1.7 Structural representation of X-IAP adapted from (Galban and Duckett, 2010)**

*X-IAP is a member of the inhibitor of apoptosis family and contains all 3 of the BIR motifs. X-IAP can directly inhibit caspases 3, 7 and 9, a process involving the use of BIR 2 and BIR 3. Interestingly, BIR 1 can complex with TAK1 and TAB1 implicating X-IAP in regulation of the NF- $\kappa$ B signaling pathway. The RING domain enables X-IAP to self ubiquitinate enabling protein levels to be regulated, at least partially, by the proteasome. The RING domain also enables oligomerization and interaction of X-IAP with c-IAP1.*

X-IAP works through directly inhibiting the caspases 3, 7 and 9, but unlike other members of the IAP family X-IAP is the only one which has been demonstrated to act as a direct competitive inhibitor binding to the caspase catalytic domain (Eckelman et al., 2006). X-IAP can also associate with Apoptosis Inducing Factor (AIF) however the exact function of this interaction is as yet unknown. Studies have demonstrated that interaction of the two

does not inhibit the ability of X-IAP to suppress the caspases, although interestingly the interaction does appear to reduce intracellular ROS levels (Wilkinson et al., 2007). However, the anti-apoptotic capability of X-IAP can be prevented by proteins containing an IAP binding motif (IBM) such as Smac/DIABLO which inhibit X-IAP through steric hindrance.

Interestingly X-IAP function is not limited to control of the caspases, it also plays an integral role in multiple other cellular functions. For example through complexing with the serine threonine kinase TGF- $\beta$ -activated kinase 1 (TAK1) and TAK binding protein 1 (TAB1) X-IAP is implicated in NF- $\kappa$ B signaling, although evidence is conflicting as to whether TAK1 is essential for X-IAP activation of NF- $\kappa$ B. Interestingly X-IAP can also bind copper metabolism domain containing 1 (COMMD1) a suppressor of the NF- $\kappa$ B pathway (Galban and Duckett, 2010).

Levels of X-IAP appear, at least in part, to be regulated by the proteasome and through its RING domain X-IAP possesses the ability to self-ubiquitinate (Yang and Li, 2000a). This is not the sole function of the RING domain as it also has a role in oligomerization (Galban and Duckett) and is involved in the interaction of X-IAP with c-IAP1 in which c-IAP1 through use of its E3 ligase RING domain marks X-IAP for proteasomal degradation (Cheung et al., 2008).

As is to be expected of any inhibitor of apoptosis, levels of X-IAP decrease in the presence of stress inducing and pro-apoptotic stimuli. It has been proposed by Henson et al. that in neutrophils activation of the ERK/MAPK pathway can protect against stress induced apoptosis by preventing a decrease in levels of X-IAP (either through inhibiting degradation or inducing XIAP synthesis.) In addition oxidants found at sites of inflammation are hypothesized to block ERK activity via activating P38 promoting a decrease in XIAP thereby enhancing apoptosis (Yang and Li, 2000b).



### **1.11 Intracellular signaling affecting granulocyte survival**

Whilst the basic structural and cellular characteristics occurring during apoptosis vary only slightly between cell types, the mechanisms controlling apoptosis differ greatly. There are key differences in the mechanisms regulating lymphocytes and granulocytes and even between types of granulocytes (Haslett, 1997, Ward et al., 1999b). It has been revealed through *in vitro* observations that inflammatory stimuli such as IL-1b, IL-2, IL-8 IL-15, TNF- $\alpha$  (>12hr incubation), IFN- $\gamma$ , GM-CSF, GCSF, LPS, C5a and hypoxic conditions all have the ability to delay neutrophil apoptosis. These pro-survival factors allow neutrophils the opportunity to persist at the site of infection ensuring efficient removal of invading pathogens. GM-CSF also acts to delay eosinophil apoptosis, while IL-5 another suppressor of eosinophil apoptosis has no effect on neutrophil life span (Ward et al., 1999b). The fate of the granulocyte at the inflammatory site can therefore be viewed in terms of a fine balance between pro-apoptotic and the anti-apoptotic signals from local pro-inflammatory mediators. Some of the pathways known to influence apoptosis will be considered.

#### **1.11.1 NF $\kappa$ B pathway and role in neutrophil apoptosis**

The role of the NF- $\kappa$ B pathway in inflammation is notoriously complex given its involvement in a plethora of inflammatory processes ranging from the pro-inflammatory functions of cell survival, leukocyte recruitment and cytokine production to the pro-resolving effects of neutrophil apoptosis. Consequently the homeostatic mechanisms in place to regulate such complexity are intricately linked and balanced.

As previously mentioned the transcription factor NF- $\kappa$ B is a key component in regulating the fate of the granulocyte as it is implicated in up-regulating anti-apoptotic genes. It is through NF- $\kappa$ B that pro-inflammatory mediators such as LPS and TNF- $\alpha$  are thought to extend neutrophil survival. The role of NF- $\kappa$ B on neutrophil and eosinophil survival has been investigated using pharmacological tools such as the NF- $\kappa$ B inhibitor gliotoxin, which when used in conjunction with exposure to LPS, was found to inhibit the anti-apoptotic effects of LPS. Additionally, at early time points gliotoxin dramatically enhances the

apoptotic action of TNF- $\alpha$  (Ward et al., 1999a). The importance of NF- $\kappa$ B in neutrophil and eosinophil survival has also been demonstrated using physiological NF- $\kappa$ B inhibitors, namely the prostaglandin D<sub>2</sub> metabolites D12 prostaglandin J2 (D12PGJ2) and 15-deoxy-D12,14prostaglandin J2 (15dPGJ2) (Ward et al., 2002). These also increased granulocyte apoptosis and prevented mediator induced survival. In addition PGD<sub>2</sub> metabolites have been shown to possess therapeutic benefits for the treatment of rat pleurisy where they were found to induce neutrophil and macrophage apoptosis to bring about resolution of acutely inflamed tissue (Gilroy et al., 2003). Although less is known about the pathways mediating eosinophil apoptosis, NF- $\kappa$ B is thought to be involved (Ward et al., 1999a, Fujihara et al., 2005, Fujihara et al., 2002). When incubated with TNF- $\alpha$ , eosinophils appear to lose cytoplasmic I $\kappa$ B- $\alpha$  allowing the released NF- $\kappa$ B to travel to the nucleus. In addition, there is evidence suggesting that TNF- $\alpha$  triggered eosinophils apoptosis may be more sensitive to NF- $\kappa$ B inhibition than constitutive apoptosis (Fujihara et al., 2002). Using a cell permeable HIV-tat linked super-repressor of the NF- $\kappa$ B inhibitor molecule I $\kappa$ B $\alpha$  we have confirmed a role for NF- $\kappa$ B in the regulation of eosinophil apoptosis as this inhibitor induced direct eosinophil apoptosis (Fujihara et al., 2005). Other groups have now verified the importance of NF- $\kappa$ B activation in the regulation of human granulocyte apoptosis (Arruda et al., 2004, Choi et al., 2003, Francois et al., 2005, Wang et al., 2003, Castro-Alcaraz et al., 2002, Vancurova et al., 2001).

However, there is a dichotomy in terms of a role for NF- $\kappa$ B inflammation where NF- $\kappa$ B also can have an anti-inflammatory/pro-resolving role during the resolution of acute inflammation (Lawrence et al., 2001). The developments of therapeutics that specifically manipulate the anti-inflammatory effects of NF- $\kappa$ B are currently being investigated. Although I $\kappa$ B $\alpha$  is an endogenous negative regulator of NF- $\kappa$ B, therapeutic administration of this molecule itself would not be suitable due to its rapid biodegradation *in vivo*. However, recent studies have shown that a stable chimeric form of this molecule, comprising the super-repressor I $\kappa$ B $\alpha$  fused to a membrane transducing domain of HIV-tat protein, can promote the resolution of inflammation in an acute rat carrageenan induced pleurisy model (Blackwell et al., 2004). Furthermore, IKK $\alpha$ , an upstream regulator of I $\kappa$ B $\alpha$  and NF- $\kappa$ B, has recently been shown to negatively regulate macrophage activation, local

inflammation and innate immunity to bacterial infections (Lawrence et al., 2005). IKK $\alpha$  inhibits NF- $\kappa$ B activity by inducing the NF- $\kappa$ B subunits RelA and c-Rel and suppressing their interaction with pro-inflammatory gene promoters. In addition, genetically modified mice that have inactivated IKK $\alpha$  have an exaggerated inflammatory response in a model of LPS-induced septic shock (Lawrence et al., 2005).

### 1.11.2 Phosphoinositide 3-Kinase (PI3K)

PI3K is another vital signaling molecule that is likely to regulate many of the pro-inflammatory and anti-apoptotic pathways triggered by the inflammatory mediators (e.g., LPS, GM-CSF and TNF- $\alpha$ ). There are three classes of the PI3K family, class I, class II and class III, of which class I PI3Ks are subdivided again according to the organization of their catalytic and regulatory subunits along with their mode of action into classes 1A and 1B. The class 1A p85/p110 isoform of PI3K appears to be involved in the delay of neutrophil apoptosis induced by LPS, GM-CSF and TNF- $\alpha$ . However, inhibition of PI3K signaling using specific PI3K inhibitors do not influence constitutive rates of neutrophil apoptosis (Lindemans and Coffey, 2004). PI3K affects apoptosis *via* controlling the activation of Akt, NF- $\kappa$ B and the cAMP response element binding protein (CREB). For example, in PI3K $\gamma$  knockout mice translocation of NF- $\kappa$ B to the nucleus and phosphorylation of CREB were decreased. This correlated with reduced expression of the anti-apoptotic proteins Bcl-XL and Mcl-1. It was also found that Akt inactivated Bad, Forkhead and GSK-3 $\beta$  through phosphorylation, with Akt now known to be an integral enzyme in neutrophil apoptosis as it controls Mcl-1 expression through CREB as well as NF- $\kappa$ B and Bcl-XL (Yang et al., 2003). PI3K is also implicated in the regulation of eosinophils recruitment and survival *in vivo*. Recent studies, using PI3K $\gamma$ -deficient mice and specific PI3K inhibitors, have demonstrated that PI3Ks critically regulate the recruitment and survival of eosinophils in a model of allergic pleurisy. PI3K inhibitors administered during post antigen challenge resulted in a significant increase in apoptotic events and clearance of eosinophils. It was concluded that PI3K $\gamma$  is necessary for maintenance of eosinophilic inflammation *in vivo* and that other isoforms of PI3K may be relevant for eosinophils recruitment (Pinho et al., 2005).

### 1.11.3 Mitogen Activated Protein Kinases (MAPK)

Upstream kinases regulate the three subfamilies of MAPKs by phosphorylation. These three types of ubiquitously expressed enzymes are p38, extracellular-signal regulated kinases (ERKs) and stress-activated protein kinase/c-jun amino-terminal kinase (SAPK/JNK); in most cell types they play a pivotal role in the regulation of survival. Inflammatory mediators activate several MAPKs therefore it is proposed that crosstalk between MAPKs may be responsible for increasing granulocyte survival. ERK pathways are known to be activated by agents that affect granulocyte responsiveness and apoptosis (e.g., GM-CSF, LPS, IL-8, IL-15 and C5a). Using MEK inhibitors (especially PD098959) it has been suggested that ERK activation is not essential for controlling constitutive apoptosis but that it is likely to be important for maintaining survival induced by certain inflammatory mediators (e.g., GM-CSF) see (Walker et al., 2003).

Our recent studies have shown that the pro-survival molecules pERK1/2 and Bcl-xL are present in inflammatory cells that are predominant at the onset of inflammation and become down-regulated during the resolution phase of the inflammatory response (Sawatzky et al., 2006). Furthermore, we have qualitatively shown that inducing granulocyte apoptosis with the ERK1/2 inhibitor PD98059 promotes the resolution of inflammation when given at the peak of the inflammatory response in a rat carrageenan-induced pleurisy *in vivo* model. This study shows that the induction of neutrophil apoptosis halts the progression of the inflammatory response and pro-actively initiates the onset of resolution.

The importance of p38 and SAPK/JNK in neutrophil survival however remains more controversial. p38 may be an important factor in stress induced apoptosis, yet is thought to have no involvement in Fas or constitutive apoptosis. In addition in GM-CSF treated neutrophils it has been reported that p38 does not play a role in increasing neutrophil survival as it fails to become phosphorylated. This is supported by the observation that inhibition of p38 by SB203580 fails to affect the anti-apoptotic capacities of GM-CSF. However, a possible role for p38 in neutrophil survival could be provided by its apparent ability to phosphorylate and thereby inhibit caspases-3 and 8 (Gardai et al., 2004, Alvarado-

Kristensson et al., 2002, Frasch et al., 1998, Sheth et al., 2001). It has been proposed that once the caspases become activated upon the neutrophil entering apoptosis they can result in the cleavage and subsequent degradation of ERK and p38. This cleavage of MAPKs is thought to be prevented by G-CSF therefore this could pose a possible pathway through which apoptosis is inhibited in neutrophils (Alvarado-Kristensson et al., 2004, Suzuki et al., 2001).

There is a lack of conclusive evidence regarding the role of JNK in neutrophil apoptosis. However, the limited data suggest that inflammatory mediators such LPS and TNF- $\alpha$  have effects on the JNK pathway to influence function. There is some debate however as to whether neutrophil apoptosis is caused by activation of JNK or if it is in fact merely a consequence of it. This is because it has been reported that MAPK kinase and MEKK-1 are cleaved when the cell undergoes apoptosis and that consequently they can activate JNK (Nolan et al., 1999, Avdi et al., 2001).

#### **1.11.4 Cyclic Adenosine Mono Phosphate (cAMP)**

Granulocyte apoptosis is also delayed through an increase in cAMP brought about via some inflammatory mediators (e.g., prostaglandins) and pharmacological agents that elevate cAMP (e.g., the cell permeable analogue db-cAMP) (Martin et al., 2001, Hallsworth et al., 1996, Peacock et al., 1999, Rossi et al., 1995, Tortorella et al., 1998). The precise mechanism of action of cAMP mediated delay of neutrophil apoptosis remains to be elucidated. It has been suggested that this effect may be mediated by both a PKA-dependent (Krakstad et al., 2004, Parvathani et al., 1998) and PKA-independent mechanism (Martin et al., 2001). Similarly cAMP elevation delays eosinophils apoptosis however the mechanisms by which this occurs still remains to be fully elucidated.

#### **1.11.5 Calcium**

Use of agents (e.g. A23187 and thapsigargin) that raise cytosolic free calcium in granulocytes have shown that this important second messenger molecule not only regulates

granulocyte function but also apoptosis. In neutrophils, raised cytosolic calcium levels have been found to delay apoptosis (Whyte et al., 1993, Cousin et al., 1997, Murray et al., 2003b) whereas increased calcium levels in eosinophils appear to enhance rates of apoptosis (Cousin et al., 1997). The reasons for such differential regulation is currently not known and it is also apparent that there is a dissociation between raised elevation of cytosolic calcium and rates of apoptosis (Murray et al., 2003b).

### 1.11.6 Glucocorticoids

Glucocorticoids are one of the major forms of treatment for inflammatory diseases and, whilst they prove invaluable especially in lymphocytic and eosinophilic dominant diseases such as asthma they appear to be less beneficial in neutrophilic-mediated inflammation. This may be because whilst glucocorticoids act to up-regulate the phagocytic ability of macrophages (Liu et al., 1999, Giles et al., 2001, Heasman et al., 2004) and increase eosinophil (Meagher et al., 1996) and lymphocyte (McConkey et al., 1989, Wyllie et al., 1984, Wyllie and Morris, 1982) apoptosis they extend neutrophil life span (Meagher et al., 1996, Cox and Austin, 1997, Cox et al., 1995) therefore slowing their removal from the site of inflammation. Sivertson K.L *et al* reported in 2007 that the differing effect of dexamethasone on neutrophils and eosinophils is, at least in part, due to differing effects on Mcl-1 (Sivertson et al., 2007). Whilst dexamethasone decreases Mcl-1 in eosinophils leading to apoptosis, it maintains expression in neutrophils through an as yet unidentified post translational mechanism. It is plausible that the same effect may occur *in vivo*.

There are two known isoforms of the glucocorticoids receptor (GR), GR $\alpha$  and GR $\beta$ , the latter of which lacks a steroid binding domain rendering it unable to bind with glucocorticoids. Whilst it is known that the predominant isoform of the glucocorticoid receptor in neutrophils is the  $\alpha$ -isoform, little is known about the glucocorticoid survival pathway in neutrophils. One theory is that the ability of glucocorticoids to induce apoptosis in a cell, revolves around the ratio of GR $\alpha$  to GR $\beta$  (Strickland et al., 2001). Interestingly it has also been shown that other steroids can also affect neutrophil apoptosis. For example,

oestradiol and progesterone have an anti-apoptotic effect on neutrophils, a response that is reversed by activating the Fas pathway (Molloy et al., 2003).

#### **1.11.7 Nitric oxide**

Nitric oxide is not only important in the processes of vasodilatation and neurotransmission as it also plays a regulatory role in granulocyte apoptosis (Ward et al., 2000, Taylor et al., 2003). Its role, however, is not straightforward, as it appears to have both the ability to promote, as well as delay granulocyte apoptosis. The general trend in neutrophils however appears to be that high concentrations of nitric oxide have a pro-apoptotic/necrotic effect possibly *via* peroxynitrite generation (Taylor et al., 2001). In addition, it is speculated that pathways that influence granulocyte survival such as NF- $\kappa$ B may be disrupted indirectly by the generation of nitric oxide (Fortenberry et al., 2001). On the contrary, low concentrations of nitric oxide favour an anti-apoptotic effect that is proposed to involve S-nitrosation of caspase enzymes or an increase in cGMP (Taylor et al., 2001).

#### **1.11.8 Miscellaneous**

There are many other mediators, drugs and agents that influence granulocyte apoptosis that have not been specifically covered in detail in this review. Others include, oxidative stress, bacteria and their products, and cytokines such as macrophage migration inhibitory factor (MIF), many of which have been described in other reviews (Ward et al., 1999b, Walker et al., 2003, Akgul et al., 2001, Simon, 2003, Hofman, 2004). For example, MIF an inflammatory cytokine that is released by monocytes/macrophages T-cells and eosinophils, delays neutrophil apoptosis *via* inhibiting the apoptotic pathway upstream of the mitochondria ultimately preventing activation of caspase-3 (Baumann et al., 2003). MIF has also been proposed to be involved in eosinophil-dependent inflammatory disorders, such as asthma. Eosinophils have been shown to be an important source of MIF and bronchoalveolar lavage fluid samples from asthma sufferers demonstrating increased levels of MIF when compared to samples obtained from healthy non-asthmatics (Rossi et al.,

1998a). It is now recognised that the inflammatory environment is hypoxic (often with a  $pO_2 < 3\%$ ).

Granulocytes function efficiently under these conditions and it is believed that adaptive responses to hypoxia are controlled mainly by the transcription factor HIF-1 $\alpha$ . Indeed it has been shown that HIF-1 $\alpha$  is essential for myeloid cell-mediated inflammation *in vitro* and *in vivo* (Cramer et al., 2003). Hypoxic conditions profoundly delay neutrophil (Hannah et al., 1995, Mecklenburgh et al., 2002, Murray et al., 2003a, Walmsley et al., 2005) and eosinophil (Ward et al., 1999b) apoptosis. The precise mechanism underlying the hypoxia-mediated neutrophil survival is unknown but good evidence suggests that HIF-1 $\alpha$ -dependent NF- $\kappa$ B activity is involved (Walmsley et al., 2005).

### **1.12 Macrophage removal of apoptotic granulocytes**

Resolution of inflammation requires that granulocytes are removed from the site of cellular damage by a process involving granulocyte apoptosis and subsequent disposal *via* phagocytosis by tissue resident macrophages or other cells with a capacity for phagocytosis (e.g., fibroblasts, endothelial cells). Importantly, there is much evidence suggesting that phagocytes when ingesting apoptotic cells increase their expression and release of anti-inflammatory pro-resolution mediators and down-regulate release of pro-inflammatory mediators (Fadok et al., 1998, Liu et al., 1999). Apoptotic cells experience changes to their cell membrane allowing recognition and ingestion of the apoptotic cells by phagocytes via binding to specific receptors either directly or via bridge molecules.

One such signal suggested to be a key mechanism behind the removal of apoptotic cells is the recognition of PS on the apoptotic cell surface by specific PS receptors. PS is located on the inner leaflet of the plasma membrane of viable cells and is maintained there by aminophospholipid transferase, however it is disruption of this transferase during apoptosis which is responsible for PS exposure (Bratton et al., 1997) along with activation of phospholipid scramblase by caspase 3 (Frasch et al., 2000). Furthermore recent research indicates that in order for PS to be recognised by the phagocyte as an engulfment signal it



must first be oxidized by cytochrome C before it is externalized (Jiang et al., 2003). However, a further theory is that PS can more accurately be described as an indicator of cell death as Brouckaert identified that PS is externalized in death ligand induced necrotic cell death, marking these cells for engulfment by macrophages (Brouckaert et al., 2004). For some cases of death receptor/ligand binding bridge molecules are required to aid the process. For example milk-fat globule epidermal growth factor 8 (MGF-E8) aids binding of the vitronectin receptor to the PS ligand (Wu et al., 2006), whilst binding of PS to the Mer kinase receptor is aided by the bridging molecule Gas6. However not all PS receptors appear to require the presence of bridge molecules, research by (Park et al., 2008) suggests the stabilin-2 receptor binds to the PS without the use of a bridging molecule.

PS is not thought to be the sole 'eat me' signal responsible for the removal of apoptotic neutrophils (see (Savill et al., 2002, Gregory and Devitt, 2004, Giles et al., 2000)). Other cell membrane recognition ligands include calreticulin, stored in the endoplasmic reticulum but which is upregulated and presented on the cell surface upon apoptosis. Interestingly it has also been postulated that DNA exposed on the cell surface can act as a ligand indicating cell death to professional phagocytes (Henson and Hume, 2006). Therefore it has been proposed that different mechanisms may be responsible for signaling removal of apoptotic cells in different tissue types. Furthermore there is also evidence for "don't eat me" signals transmitted by viable leucocytes in the form of homophilic ligation of CD31, which repulses macrophages. This homophilic ligation of CD31 is lacking in apoptotic leucocytes enabling tight binding and engulfment of the apoptotic cell by the macrophage (Brown et al., 2002).

Interestingly, it has been suggested that apoptotic cells also have the capacity to liberate specific agents that promote the recruitment of cells capable of their removal (Lauber et al., 2003). For example upon apoptosis the enzyme aminoacyl-tRNA synthase is processed into two products. One of these products which is released from the dying cell is endothelial monocytes-activating polypeptide II (EMAP II) which mediates the infiltration of leucocytes and monocytes to the inflammatory loci by acting as a ligand for the interleukin 8 receptor (IL-8), in addition EMAP II also stimulates TNF- $\alpha$ , myeloperoxidase and tissue

factor (Wakasugi and Schimmel, 1999). Other molecule expressed by apoptotic cells which possesses chemotactic properties for monocytes/macrophages is the lipid mediator sphingosine-1 phosphate (S1P) (Gude et al., 2008) and lysophosphatidylcholine (Mueller et al., 2007). The chemokine CX<sub>3</sub>CL1 which is released from apoptotic lymphocytes also acts as an attractant for mononuclear phagocytes (Truman et al., 2008).

Just as it is important for the apoptotic cells to express factors which stimulate migration of phagocytes towards them it is also essential if effective resolution of inflammation is to be achieved that granulocyte migration to the site of inflammation is inhibited. This is necessary to prevent an inundation of apoptotic granulocytes, which would over burden the phagocytes resulting in the excess apoptotic granulocytes entering secondary necrosis. Therefore apoptotic cells secrete lactoferrin, which has been demonstrated to significantly inhibit granulocyte migration both *in vitro* and *in vivo* (Bournazou et al., 2009) thus allowing the release of molecules to attract phagocytes without attracting further granulocytes (e.g. the release of nucleotides) (Elliott et al., 2009).

### **1.13 Resolution of inflammation**

During the resolution of the inflammatory response there is a delicate inter-related balance between the leucocytes, disruption of which can have catastrophic effects, ultimately resulting in the non-resolving inflammation seen in inflammatory disorders such as chronic obstructive pulmonary disease (COPD) and Cystic Fibrosis (CF).

COPD is a term that is used to define a number of conditions such as emphysema and chronic bronchitis in which airflow to the lungs is limited by an abnormal inflammatory response, triggered by noxious particles such as cigarette smoke, leading to increased inflammation and susceptibility to infections. The disease is usually progressive and once an airway obstruction has become established a return to full previous function is not possible (Plataki et al., 2006). Symptoms of COPD include dyspnea, rhonchi, chronic coughing and sputum production, and in advanced cases COPD can lead to respiratory failure. Neutrophils are key effector cells in the pathogenesis of COPD, accumulating in the

airways of sufferers. Furthermore, large numbers of activated neutrophils are found in bronchoalveolar lavage fluids from patients with COPD (both during exacerbations and stable phases of the disease) (Pauwels et al., 2001). The driving force behind this neutrophilic inflammation is thought to be activation of respiratory macrophages by noxious particles, increasing their production of neutrophil chemoattractants such as IL-8. Upon activation neutrophils release proteases (e.g. elastase) exacerbating bronchitis through the stimulation of mucus production, and emphysema through remodelling of the lung parenchyma (Barnes, 2010).

It remains to be elucidated if the COPD neutrophil exhibits a propensity to evade apoptosis. Pletz et al concluded in 2004 that circulating neutrophil apoptosis is inhibited during exacerbations of COPD compared to neutrophils from healthy volunteers, however, upon remission normal rates of apoptosis appeared to be returned (Pletz et al., 2004). This was supported by Milara et al who observed a non significant decrease in apoptosis of circulatory neutrophils from patients with early onset COPD (Milara et al., 2012). Whereas, when differences between circulating neutrophils from healthy volunteers, smokers and COPD patients were investigated by Noguera et al in 2004 they reported similar rates of neutrophil apoptosis between all groups. Interestingly, they observed that apoptotic neutrophils from COPD patients appeared to be activated to a greater extent than those from the other conditions, exhibiting decreased levels of CD62L and increased levels of CD11b implicating that the COPD neutrophil possesses the potential ability to exacerbate the inflammatory response even when undergoing apoptosis, furthermore, increased expression of CD11b may impact on macrophage recognition of the apoptotic cell (Noguera et al., 2004). Limitations of these studies are that circulating neutrophils were examined and these would not be subjected to the inflammatory processes under way in the COPD lung (Plataki et al., 2006). Ryttilä et al in 2006 could not find evidence of decreased neutrophil apoptosis in sputum samples from patients with COPD compared to healthy smokers or healthy non-smokers despite the samples from COPD patients and smokers having significantly higher numbers of neutrophils (Ryttilä et al., 2006); although as pointed out by Persson and Uller getting an insight into the relationship between apoptosis and phagocytosis in lumen cell samples would be complicated due to the non migratory nature of apoptotic cells and

further investigation into neutrophil apoptosis in diseased mucosal tissues would be beneficial (Persson and Uller, 2012).

Whilst the mechanism and impact of evasion of neutrophil apoptosis in COPD remains controversial, defects in the apoptotic process of neutrophils have been demonstrated to play a pivotal role in Cystic Fibrosis. Cystic Fibrosis is one of the most prevalent genetically inherited diseases, with approximately 1 in 20 Caucasians being heterozygous for one of the Cystic Fibrosis trans-membrane conductance regulator gene mutations. It is a devastating disease which results in persistent neutrophilic inflammation, repeated respiratory infections and ultimately respiratory failure (Hayes et al., 2011). CFTR mutations have been demonstrated to have an anti-apoptotic effect on epithelial cell lines (Gottlieb and Dosanjh, 1996, Jungas et al., 2002) and perturbations in the apoptotic mechanisms of CF neutrophils have been observed providing a possible explanation for the prevalence of neutrophils in CF associated respiratory infections. Circulatory neutrophils from CF patients were found to have reduced expression of the TNF- $\alpha$  death receptor TNF-1 possibly indicating decreased efficiency of the extrinsic apoptotic pathway in CF neutrophils (Downey et al., 2007, Hayes et al., 2011). An increase in the actin binding protein coronin-1 was also found in neutrophils from CF patients and was associated with an inhibition of apoptosis (Moriceau et al., 2009, Hayes et al., 2011). Furthermore, inhibition of CF neutrophil apoptosis appears to be a phenomenon common to all CFTR mutations and not always dependent on whether or not an infection is present. Interestingly delayed neutrophil apoptosis was also observed in CFTR heterozygotes, with normal rates of neutrophil apoptosis being restored upon administration of R-roscovitine or the thiol-oxidising agent diamide, implying that neutrophil survival in CF patients is related to the CFTR mutation and is not simply a consequence of chronic infection (Moriceau et al., 2010).

If tissue homeostasis is to be restored to the inflammatory loci it is essential that neutrophil recruitment is halted and that apoptotic neutrophils are removed in a safe and controlled manner through engulfment by professional phagocytes. Apoptotic neutrophils act to halt the influx of neutrophils by releasing lactoferrin and the presence of protectins and

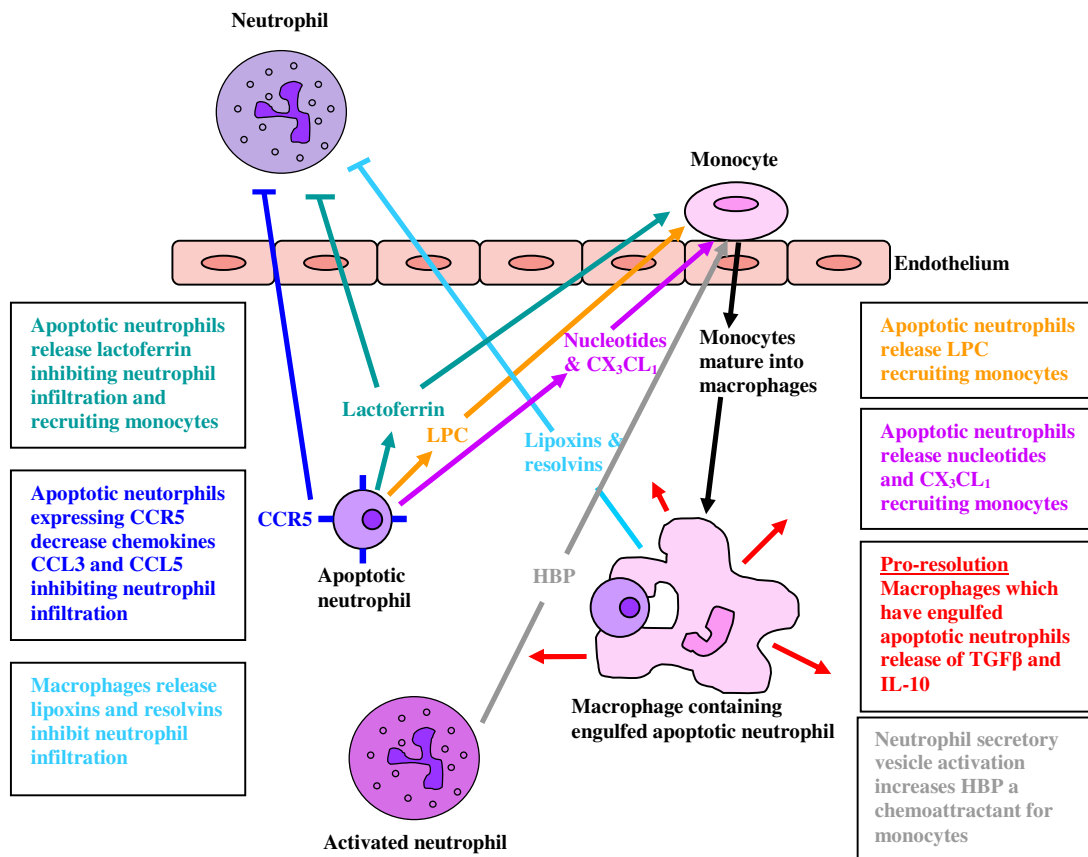
resolvins upregulates the expression of CC-chemokine receptor 5 (CCR5) on apoptotic neutrophils. This decreases levels of CCR5-binding chemokines such as CCL3 and CCL5 which have a chemotactic influence on viable neutrophils. Monocyte influx to the inflammatory milieu is also increased by apoptotic neutrophils through release of lysophosphatidylcholine (LPC), nucleotides which increase monocytes recruitment through P2Y2 and, CX<sub>3</sub>C-chemokine ligand 1 (CX<sub>3</sub>CL<sub>1</sub>) attracting monocytes by their CX<sub>3</sub>C-chemokine ligand receptor 1 (CX<sub>3</sub>CR<sub>1</sub>). Macrophages also contribute to the inhibition of neutrophil recruitment through release of lipoxin and resolvins lipid mediators, which in addition also increase the influx of monocytes. Furthermore once the macrophages have removed the apoptotic neutrophils via phagocytosis transcription of pro resolution genes is activated initiating the liberation of interleukin 10 (IL-10) and transforming growth factor- $\beta$  (TGF $\beta$ ) (Soehnlein and Lindbom, 2010) see Figure 1.9.

Fascinatingly it appears that the relationship between apoptotic neutrophils and their engulfment by professional phagocytes could provide a mechanism for homeostatic regulation of granulopoiesis. Once apoptotic neutrophils have been engulfed by macrophages and dendritic cells the professional phagocytes decrease secretion of IL-23. IL-23 controls the production of IL-17 by neutrophil regulatory T cells therefore a decrease in IL-23 leads to a subsequent decrease in IL-17 production. IL-17 in turn regulates G-CSF production and therefore granulopoiesis. A decrease in IL-17 results in a decrease in G-CSF and subsequently a reduction in granulopoiesis and neutrophil production (Stark et al., 2005).

### **1.13.1 Resolution of inflammation: the role of the neutrophil**

It was once thought that the reduction in neutrophils at the inflammatory loci during resolution of inflammation occurred via a passive process where the pro-inflammatory mediators in the inflamed tissue simply became diluted removing the chemotactic gradient that draws the neutrophils. However there is now a wealth of evidence that this process is far from passive. Upon completing its defensive mission the neutrophil can switch from its pro-inflammatory phenotype, defined by the release of chemoattractants such as LTB<sub>4</sub>

(synthesised from arachidonic acid) to an anti-inflammatory pro-resolution phenotype in which fatty acids are instead synthesised into resolution promoting lipoxins (derived from arachidonic acid), resolvins (from omega-3 fatty acids) and protectins (from the omega-3 fatty acid docosahexaenoic acid). The release of which halts the influx of viable neutrophils and attracts macrophages to remove apoptotic neutrophils in a safe, pro-resolving manner (Serhan et al., 2008).



**Figure 1.8 Interaction between leucocytes during resolution of inflammation**

The above diagram represents some of the ways in which leucocytes inter-relate during the resolution of inflammation. For example, apoptotic neutrophils inhibit neutrophil infiltration by expressing CCR5; they also release lactoferrin, LPC, nucleotides and CX<sub>3</sub>CL<sub>1</sub> all of which recruit monocytes. Activated neutrophils also draw monocytes to areas of inflammation through an increase in HBP. Once at the inflammatory foci monocytes mature to macrophages and remove apoptotic neutrophils. Phagocytosis of apoptotic neutrophils triggers macrophages to release lipoxins and resolvins which further inhibits neutrophil recruitment.

### **1.14 Non resolving inflammation**

As is often the case with events which are as complex and intricately linked as inflammation, dysregulation of the delicate process of resolution can have a dramatic impact on the host organism. Failure of timely and complete resolution of inflammation is known to contribute to the progression and pathogenesis of an extensive list of chronic disorders such as asthma, neurodegenerative disease, inflammatory bowel disease, multiple sclerosis, chronic obstructive pulmonary disease, rheumatoid arthritis, cancer, atherosclerosis and obesity. In such incidents it is the inflammatory response of the host organism which is the main cause of tissue damage and not infection from the pathogen (Nathan and Ding, 2010).

A pertinent contributor to the aforementioned paradigm of non resolving inflammation is the neutrophil. During the efficient resolution of inflammation the neutrophil, once it has overcome the infectious agent, must be timely removed from the site of inflammation. The neutrophil enters apoptosis, exposing specific phospholipid and glycolipid signals on its cell membrane, which prompt professional phagocytes, namely the macrophages to engulf the apoptotic neutrophil (Haslett et al., 1990). This in turn converts the macrophages to a pro-resolution phenotype. As previously mentioned failure of the neutrophil to enter apoptosis in a timely manner or failure of phagocytic removal of the apoptotic cells can lead to the exacerbation of many inflammatory diseases. This has been eloquently demonstrated by Uysal *et al.* 2009, they showed that upon apoptosis or necrosis neutrophils release the enzyme cytosolic peptidyl arginine deiminase which when in the presence of high calcium (present in the neutrophil cytoplasm during both types of cell death) becomes activated and can citrullinate joint proteins such as fibrinogen and collagen. These citrullinated proteins have been found to react with antibodies found in the synovial fluid of rheumatoid arthritis patients, which are rarely present in healthy individuals (Uysal et al., 2009). Therefore an abundance of apoptotic and necrotic neutrophils can exacerbate this reaction which in turn recruits more neutrophils forming a vicious circle of destruction (Nathan and Ding, 2010).

### 1.15 Targets for pharmacological intervention

If this process of resolution is defective, it is believed that tissue damage can ensue due to the accumulation of apoptotic cells and subsequent liberation of histotoxic contents. Manipulation of the resolution phase of the inflammatory process is a promising area to provide novel anti-inflammatory therapies, selectively accelerating apoptosis in specific inflammatory cells and enhancing their subsequent clearance by exploiting their differences in intracellular regulation. Inhibition of the pro-survival signals found at the inflammatory site (for example GM-CSF and IL-5) and selective induction of specific receptors such as Fas to bring about apoptosis in chosen inflammatory cells would increase the rate of apoptosis at inflammatory sites thereby aiding the resolution process. Another optimistic area for pharmacological intervention is the regulation of the uptake of the apoptotic cells by phagocytes. It has been demonstrated that the ability of macrophages to internalize apoptotic cells can be adjusted, for example by using prostaglandins such as PGE<sub>2</sub> to elevate macrophage levels of cAMP, thereby activating PKA and transforming adhesion patterns. Macrophages treated as such have a reduced capacity to uptake apoptotic neutrophils therefore implying a possible link between adhesion and macrophage phagocytic abilities (Rossi et al., 1998b). Furthermore the internalization of apoptotic neutrophils by macrophages can be upregulated via ligating the transmembrane adhesion receptor CD44 (Hart et al., 1997). This molecule has been found to be of particular importance in the resolution of lung inflammation (Teder et al., 2002). Other means which have also been found to alter macrophage ability to internalize apoptotic cells are; by activating macrophage signaling pathways (e.g., PKC) (Hu et al., 2002, Todt et al., 2004, Todt et al., 2002), ligating extracellular matrix receptors (Hart et al., 1997, Hart et al., 1998), use of certain cytokines (e.g., IL-10, GM-CSF, TGF- $\beta$ ) (Ren and Savill, 1995, Ogden et al., 2005, Godson et al., 2000, Mitchell et al., 2002) or eicosanoids (e.g., lipoxins). Recent breakthroughs regarding the ability of glucocorticoids to increase the phagocytic potential of macrophages to remove apoptotic cells by innocuous non-inflammatory means have proved invaluable in aiding the understanding of their action (Liu et al., 1999, Heasman et al., 2004, Giles et al., 2001, McColl et al., 2009, McColl et al., 2007). This could be linked to studies revealing that glucocorticoids have the ability to



transform the cytoskeletal organization and the protein generating ability of monocytes driving them to become highly phagocytic macrophages (Giles et al., 2001). Interestingly, it has been proposed that the cytokine environment present at the site of inflammation may influence the effectiveness of glucocorticoids having implications on their therapeutic use. For example, IFN- $\gamma$  has the ability to counteract the increased phagocytic ability of macrophages obtained from glucocorticoid treated monocytes without altering their morphology (Heasman et al., 2004). This increase in the phagocytic ability of macrophages through exposure to glucocorticoids complements glucocorticoids capacity to up-regulate eosinophil and lymphocyte apoptosis, possibly explaining why the use of glucocorticoids proves to be a successful treatment for a range of inflammatory diseases.

### **1.16 Therapeutic applications and drug discovery**

The safe engulfment and removal of apoptotic cells is an emerging target for the treatment of inflammatory diseases. Indeed, this mechanism has now been attributed to many of the drugs that are already in clinical use, such as the glucocorticoids and NSAIDs. Glucocorticoids have been shown to induce apoptosis of inflammatory cells (except neutrophils), and importantly, to promote phagocytosis of these effete cells ensuring their safe removal thus limiting tissue damage and injury. In addition, there are many endogenous 'brake' mechanisms that halt inflammation and actively drive the resolution of inflammation. These include the cyclopentenone prostaglandins, NF- $\kappa$ B p50/50, lipoxins/resolvins, annexin 1 (formerly known as lipocortin 1) and caspases, as previously discussed in this review. These have been shown to induce apoptosis and/or phagocytosis in addition to their other known anti-inflammatory properties (Gilroy et al., 2004, Gilroy et al., 2003). Stable analogues and structurally related analogues of cyclopentanone prostaglandins and lipoxins show some promise in various *in vivo* models of inflammation and may prove useful in the treatment of non-resolving inflammatory disorders, such as arthritis, psoriasis and asthma (Gilroy et al., 2004). There are now a number of novel anti-inflammatory strategies under development that target the regulation of apoptosis. In particular there are promising drugs that interfere with caspase activation to influence apoptosis already in phase I and phase II clinical trials for the treatment of rheumatoid

arthritis, sepsis, hepatitis and stroke (Murphy et al., 2003a, Murphy et al., 2003b, Reed, 2002).

The forced selective induction of apoptosis is thought to be beneficial for the treatment of inflammatory diseases due to a variety of reasons. Primarily, the removal of potentially cytotoxic cells in a safe manner that maintains membrane integrity prevents potential damage to surrounding tissues. Also, phagocytosis of apoptotic cells leads to the generation of anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$ 1 and also may have other unknown endogenous pro-resolving mechanisms that aid resolution of inflammation. Indeed, previous research has shown that the exogenous administration of apoptotic cells induces resolution in an experimental model of inflammation (Huynh et al., 2002). Interestingly, autologous administration of apoptotic cells has also been clinically proven to prevent heart, kidney and lung transplant rejection (Morelli and Thomson, 2003) and limit the need for immunosuppressive drugs, which have many associated side effects and weakens the patient's immune system to kill invading pathogens and bacteria. Recent research shows that the engulfment of the apoptotic cells induces tolerogenic changes of immature dendritic cells, which then stimulates inhibitory T regulatory cells to suppress immune rejection (Lamioni et al., 2005). This clearly shows that the administration of apoptotic cells and their safe engulfment in humans has a role in the adaptive and the innate immune system by improving tolerance and promoting the resolution of inflammation.

Autoimmunity may occur due to the failure to clear apoptotic cells resulting in necrosis with the loss of cell membrane integrity, which causes the release of cytotoxic contents that initiates damage to surrounding tissues. This has been shown in approximately half of the patients with systemic lupus erythematosus where failed clearance leads to the prevalence of apoptotic cells, which has been correlated with disease severity (Mevorach, 2003). It would be beneficial to assess if the clearance defects of the phagocytic cells could be overcome in order to promote the safe removal of these unwanted cells and what outcome this strategy could have on limiting disease progression or give an improved prognosis for these patients. It is thus becoming clearer that the induction of apoptosis of inflammatory cells could be a beneficial target for the treatment of inflammatory diseases, not only to ensure

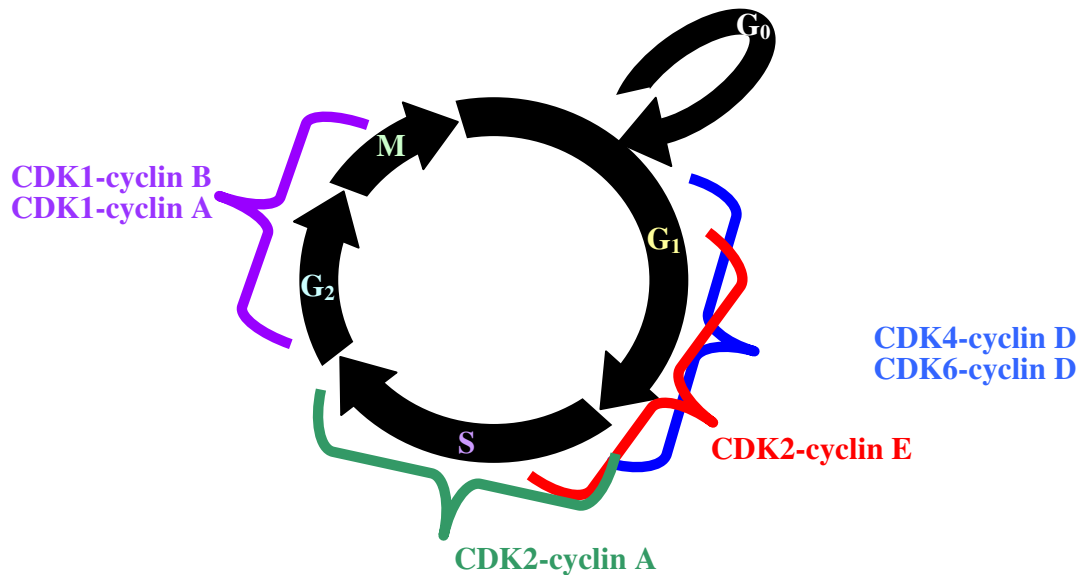
their safe removal preventing tissue damage but also to elicit an anti-inflammatory cascade of events that promote the resolution of inflammation.

### **1.17 Cyclin-dependent kinases**

An exciting new area of research in the field of apoptosis is the role of the CDKs. This is because the processes of cell death, proliferation and cell survival are all intricately linked by common pathways / molecules which have evolved, to some extent, as a safety mechanism to help prevent potentially harmful mutations in key pathways from becoming established. This is because if the mutation effects more than one process it is more likely to be noted and either repaired or for the cell to enter apoptosis and be safely removed by the professional phagocytes. For this reason we believe that CDKs, originally thought to be exclusive to cell cycle regulation and proliferation are also pivotal to the apoptotic process.

CDKs are a family of serine/threonine protein kinases, so named as they because of their interaction with the cyclins regulating the transition between phases of the cell cycle. The cell cycle is divided into the G1, S, G2, M and G0 phases. In G1 the cell commences the process of division and once the restriction point is passed at approximately 8-10 hours the cell is committed to the cycle, if the restriction point is not passed the cells enter the G0 phase where they reside in a quiescent state. During this phase the cyclins and cyclin dependent kinases are no longer present and the cell cycle machinery is dismantled. However if the cell progresses through G1 the G1/S checkpoint must be passed in order to progress onto the S phase in which DNA is synthesized, if they fail they will be committed to apoptosis. From here the cell proceeds through the G2/M checkpoint where chromosome arrangement and DNA duplication is checked and again if faults are present the cell will enter apoptosis. The M phase is the stage in which mitosis occurs, depending on if the cell passes the spindle checkpoint which ensures the chromosomes are correctly attached to the spindles (Sridhar et al., 2006, Knockaert et al., 2002). The CDKs are positively regulated via association with the cyclin and by a CDK activating kinase phosphorylating the threonine on the T-loop. The level of CDKs stay relatively constant throughout the cell cycle whereas levels of cyclins are phase dependent fluctuating due to degradation and de

novo synthesis. To date there have been 25 cyclins and 13 CDKs identified (Meijer and Raymond, 2003, Leitch et al., 2009) (please see figure 1.9).



**Figure 1.9 Schematic representation of CDK/cyclin complexes involved in the eukaryotic cell cycle.**

*The above diagram shows the eukaryotic cell cycle, which can be split into 5 phases: G<sub>0</sub> (quiescent phase), G<sub>1</sub> (the cell commences the process of division), S (DNA synthesis), G<sub>2</sub> (Chromosome arrangement checked) and M (Mitosis). The CDKs and their associated cyclins regulate transition between the phases.*

As previously mentioned CDKs sole function is no longer considered to be regulation of the cell cycle: CDKs 1 and 2 have a minor role in transcription and there is also some debate over their implication in the process of apoptosis (Golsteyn, 2005), for example, inhibition of CDK2 has been associated with a decrease in Mcl-1 and decreased phosphorylation of RNA Pol II in large B cell lymphoma cells (Faber and Chiles, 2007); CDKs 4 and 6 have been linked to the inflammatory process, for example, Liu et al demonstrated that leukocyte recruitment and adhesion is inhibited in CDK4 knockout mice with bleomycin induced lung injury (Liu et al., 2008), furthermore, a specific inhibitor of CDK4/6 exhibits resolution of inflammation in models of rheumatoid arthritis (Sekine et al., 2008); CDK5, whilst it is not thought to be involved in regulation of the cell cycle, has

a pivotal role in neurons (e.g. neuronal migration) and interestingly has been implicated in GTP-dependent granular secretion in neutrophils (Rosales et al., 2004); CDKs 7 and 9 are key players in the regulation of transcription, please see 1.17.2 for more information. Less is known about the remaining CDKs [CDK3 (involved in G0 exit), CDK8 (also implicated in transcription), CDK10 (required for passage from G2 to M) and CDK11 (potentially implicated in transcription)] and these provide interesting areas for further study (Leitch et al., 2009).

In turn, levels of CDKs are regulated by endogenous CDKis. These, like the cyclins, fluctuate in concentration providing control over the more constant CDKs. They can be subdivided into two main families Cip/Kip (p21, p27, p57) and Ink4 (p15, p16, p18, p19), with the Ink4 family enabling arrest of the cell cycle at G1 through inhibition of the CDK4-cyclin D and CDK6-cyclin D complexes through competitive inhibition of the CDK binding site, whilst the Cip/Kip family also lead to arrest of the cell cycle at G1 only through inhibition of the CDK2-cyclin E complex. In addition members of the Cip/Kip family are also implicated in the regulation of apoptosis as in certain cells p21 has the ability to sequester pro-caspase 3, and p21 can also be induced by the TGF- $\beta$  and STAT pathways having a negative effect on apoptosis induced by TGF- $\beta$  (Leitch et al., 2009).

### **1.17.1 The role of CDKs in granulocytes**

Given that it is widely regarded that neutrophils are terminally differentiated cells which are at rest in G0 there appears to be a lack of requirement for CDKs in the traditional cell cycle regulatory role. Before the effects of CDKi on neutrophils had been investigated it was discovered that CDKi prevented apoptosis in terminally differentiated neurons (Monaco and Vallano, 2003). If this were to be extrapolated to neutrophils it would be anticipated that CDKi may exert a similar effect halting apoptosis. However, our laboratory observed that conversely to the effect observed in neurons, the CDKi R-roscovitine was found to significantly increase rates of neutrophil apoptosis (Rossi et al., 2006) and eosinophils apoptosis, which are also terminally differentiated cells (Duffin et al., 2009).

Whilst the presence of CDK1, CDK2 (Rossi et al., 2006) and CDK5 (Rosales J.L., *et al.* 2004) has been confirmed in neutrophils, CDK1 and CDK2 do not appear to be direct targets for degradation during either constitutive or pharmacologically triggered neutrophil apoptosis. The reasoning behind this that there is no alteration in levels of protein expression of CDK1 or CDK2 between freshly isolated, aged, GM-CSF treated or R-roscovitine treated neutrophils (Rossi et al., 2006). Furthermore this is supported by the levels of CDKs remain fairly constant throughout the cell cycle, with regulation occurring through the fluctuation in levels of the cyclins and the cells endogenous CDKi (Knockaert et al., 2002). Interestingly whilst the levels of CDK1 and CDK2 remain constant throughout neutrophil apoptosis, when neutrophils were exposed to the Fas activating (pro-apoptotic) antibody CH11 a distinct decrease in the functionality of CDK1 occurs prior to apoptosis (Rossi et al., 2006) implicating the importance of the CDKs in neutrophil longevity. As is evident in Figure 1.9 CDK5 is not thought to play a role in regulation of the eukaryotic cell cycle, although it has been found to phosphorylate pRb, and thus is not regulated by the cyclins. Instead CDK5 is implicated in the nervous system binding to the regulatory proteins p35 and its homologue p39 (Sridhar et al., 2006). However, its presence in neutrophils opens up the possibility that CDK5 may also be implicated in neutrophil apoptosis through as yet to be identified regulatory partners.

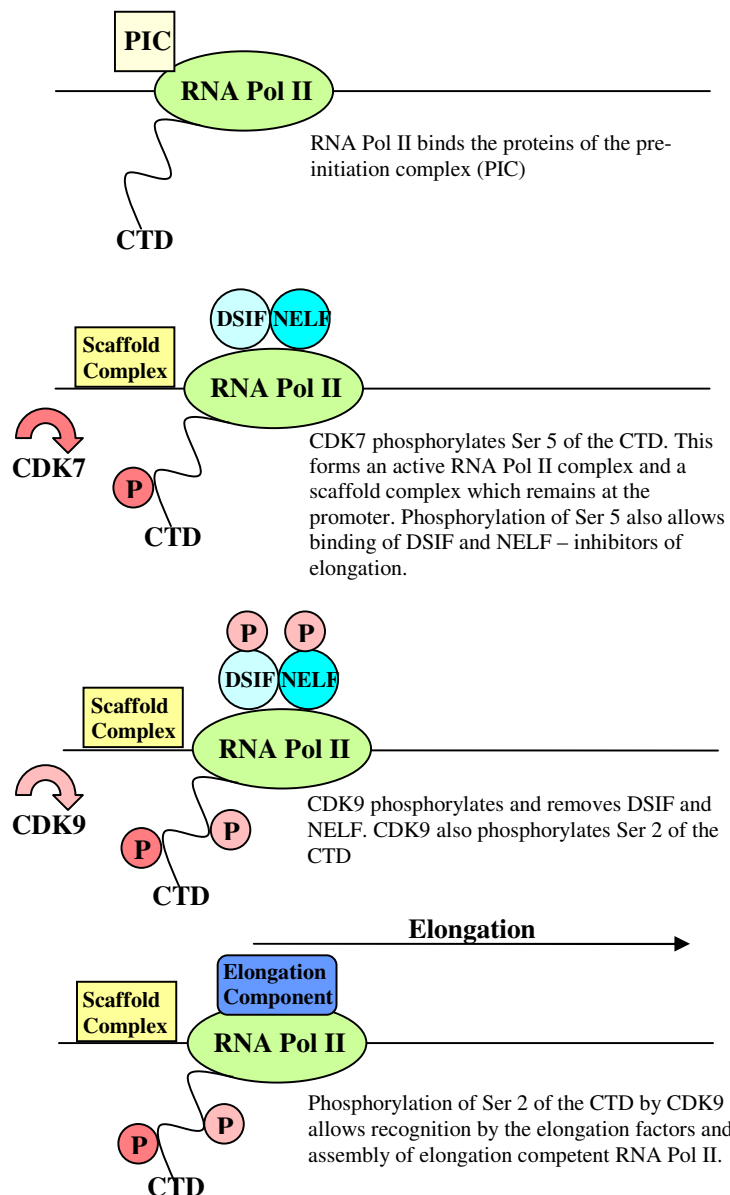
### **1.17.2 The role of CDKs in transcription**

Another important cellular function in which CDKs1, 2, 7, 8 and 9 have been implicated is the modulation of transcription through phosphorylation of RNA Pol II (Leitch et al., 2009). During the process of RNA Pol II dependent transcription the carboxyl-terminal domain (CTD) of the largest catalytic subunit of RNA Pol II can be reversibly phosphorylated. This enables the CTD to bind a range of factors essential for not only transcription but also mRNA modifications such as capping, splicing, cleavage and polyadenylation (Hirose and Manley, 2000).

As is depicted in Figure 1.10 phosphorylation of the CTD is critical as it regulates mRNA production. Two of the key sites for phosphorylation are serine 5 and serine 2, which can be

phosphorylated by CDK7 and CDK9 respectively. Phosphorylation of serine 5 by the CDK7 subunit of TFIIH forms an active RNA Pol II complex allowing transcription initiation, activation of factors which cap the 5' end of the nascent RNA (Ho and Shuman, 1999) and enables binding of the inhibitors of elongation DSIF and NELF (Wada et al., 1998). As a result of this the CDK9 containing positive elongation transcription factor (P-TEFb) can phosphorylate serine 2 of the CTD (Peterlin and Price, 2006). CDK9 also phosphorylates for removal DSIF and NELF both of which are essential for recognition by the elongation factors and assembly of the elongation competent RNA Pol II (Ivanov et al., 2000, Kim and Sharp, 2001, Fujinaga et al., 2004).

Perturbation of these essential phosphorylations would lead to inhibition of RNA synthesis and thus a decrease in cellular levels of the protein which it is translated into. This would have particular impact on proteins which are subject to rapid turn over, such as Mcl-1, which has a notably short half life for both its mRNA and protein. Given that CDKs 1, 2, 5, 7 and 9 fall within the inhibitory range of R-roscovitine it is highly probable that in cells exposed to this trisubstituted purine analogue transcription by RNA Pol II will be disrupted (Farahi et al., 2011). This has been eloquently portrayed by MacCallum D.E *et al.* in 2005 who demonstrated that phosphorylation of serine 2 and serine 5 of the CTD domain on RNA Pol II in myeloma cells was prevented by exposure to R-roscovitine. Consequently mRNA and protein levels of Mcl-1 decreased inducing apoptosis (MacCallum et al., 2005).



**Figure 1.10 Diagrammatic representation of the role of CDK7 and CDK9 in eukaryotic transcription**

*CDK7 is required in eukaryotic transcription as it phosphorylates Ser 5 on the CTD of RNA Pol II. This forms an active RNA Pol II complex and a scaffold complex. It also allows binding of the inhibitors of elongation. CDK9 is responsible for removal of the inhibitors of elongation and also for phosphorylation of Ser 2 of the CTD allowing recognition by elongation factors and assembly of elongation competent RNA Pol II.*



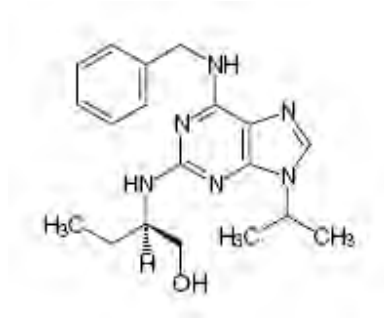
### 1.18 Pharmacological CDK inhibitors

The first specific inhibitors of CDKs were discovered in the 1990's during the hunt for novel anti-cancer treatments due to the postulation that by halting mitosis replication of mutant cells could be prevented. Until then the only inhibitors identified which successfully attenuated CDK function were non specific kinase inhibitors such as Staurosporine from *Streptomyces*, a potent inhibitor of phospholipid/calcium-dependent protein kinases. However, upon the discovery of the ability of the purine analogue olomoucine to target kinase inhibition to the CDKs (although some action against MAPKs was still present) a family of 2,6,9 trisubstituted purine CDKi came to light, of which there are approximately 50 members (Meijer and Raymond, 2003, Bach et al., 2005).

<u>Non Specific</u>	<u>Specific to CDK 1,2,5 and 7</u>	<u>Specific to CDK 4,6</u>
Flavopiridol	Olomoucine	Fascaplysin
	R-roscovitine	PD0183812
	Purvalanol B	

**Figure 1.11** Table showing the specificity of some of the purine analogue CDK inhibitors

### 1.19 Effects of R-roscovitine on neutrophils



**Figure 1.12** Structural representation of R-roscovitine

The best studied of the purine analogue CDKis is R-roscovitine, which *in vitro* has been demonstrated to decrease the proliferation index of 19 cancers (McClue et al., 2002). Other CDKis currently in clinical trials as potential novel cancer therapies are PD0332991, which is highly specific for CDK4 and CDK6; the pan-CDK inhibitor BAY1000394 and SCH727965 which inhibits CDKs 1, 2, 5 and 9. For more information please see Chapter 3.1

Whilst R-roscovitine is widely regarded as a selective inhibitor of CDKs 1, 2, 5, 7 and 9 cyclin dependent kinases are not the sole targets of its inhibitory abilities (Farahi et al., 2011). It has been demonstrated through tests on an extensive panel of kinases, identification of binding partners in various tissues by affinity chromatography and the effects of R-roscovitine on cells devoid of its target CDK 2 that R-roscovitine is not specific for CDKs alone. Although it is not active on a wide range of cellular kinases other targets for R-roscovitine include pyridoxal kinase, which activates vitamin B<sub>6</sub>, and protein kinases ERK1 (extracellular-signal related kinase 1), ERK2 and GSK3 (Knockaert et al., 2002, Bach et al., 2005).

The notion that R-roscovitine could be producing some of its varied potential therapeutic benefits in disorders ranging from glomerulonephritis to neurodegenerative diseases through inhibition of other ATP-binding kinases apart from the CDKs can not be fully disregarded. However there is compelling evidence to support the theory that R-roscovitine is exerting its beneficial effects, at least in some instances, through inhibition of the CDKs. Notably the observation that structurally distinctive inhibitors of CDKs exert comparable effects (Knockaert et al., 2000).

As the ERK pathway can be activated by inflammatory stimuli such as GM-CSF, LPS, IL-8, IL-15 and C5a to influence granulocyte longevity and responsiveness, it is essential to consider the possibility that CDKi could be exerting, at least partially, some of their pro-apoptotic, pro-resolution properties through inhibition of ERK and not the CDKs. Especially given that use of the ERK1/2 inhibitor PD98059 has implicated that ERK, whilst it is redundant in the initiation of constitutive apoptosis, does appear to play an important

role in maintaining neutrophil longevity induced by mediators such as GM-CSF (Walker et al., 2003). Furthermore research by Sawatzky D.A. *et al*, 2006 (Sawatzky et al., 2006) demonstrated that pERK1/2 and Bcl-XL are down regulated during resolution of inflammation. Also the administration of PD98059 at the peak of the inflammatory response induces granulocyte apoptosis in carrageenan-induced pleurisy models of acute inflammation. Although the previous provides a strong argument for the involvement of the ERK pathway in the resolution of inflammation it is unlikely that R-roscovitine and other CDKis are implementing their apoptosis inducing ability in neutrophils by the ERK pathway. This is because of the differing effects of CDKi and ERK inhibitors on neutrophil apoptosis. Whilst R-roscovitine has been convincingly demonstrated to elicit apoptosis in neutrophils PD98059 has not. Another interesting observation supporting this that unlike in vascular smooth muscle cells where R-roscovitine inhibits ERK activation by angiotensin II (Rosales and Lee, 2006) in neutrophils the effect of R-roscovitine on LPS-induced ERK phosphorylation is notably decreased compared to PD98059 implicating that the R-roscovitines ability to induce neutrophil apoptosis is probably not caused by diminished ERK phosphorylation. Furthermore R-roscovitine does not inhibit the ability of LPS and TNF- $\alpha$  to activate ERK, however it can over-ride survival signals which are known to activate the ERK pathway (Leitch et al., 2010). A more likely explanation for this phenomenon is that R-roscovitine elicits its effects downstream of direct inhibition of ERK (Sawatzky et al., 2006) in such a way that dominates events upstream.

The most recent advances in increasing the specificity of R-roscovitine are through the development of novel bioisosteres, where atoms or groups of atoms are exchanged for other similar atoms. Four of these R-roscovitine bioisosteres, pyrazolo[1,5- $\alpha$ ]-1,3,5-triazines, pyrazolo[1,5  $\alpha$ ]pyrimidines, pyrazolo[1,5  $\alpha$ ]pyridines and pyrazolo[4,3 d]pyrimidines have recently been reported by Jorda et al to have superior biological functions compared to R-roscovitine (Jorda et al., 2012) and these provide an exciting avenue to pursue in the hunt for effective new targeted treatments for neutrophil dominant inflammatory disorders.

## **1.20 Hypothesis and Aims**

### **Hypothesis**

The main hypothesis investigated in this thesis is that the cyclin-dependent kinase (CDK) inhibitor drugs (especially R-roscovitine) can efficaciously induce apoptosis in human neutrophils through regulation of critical survival proteins.

### **Aims**

The aim of this thesis was to further understand the mechanism through which the CDK inhibitor R-roscovitine induces apoptosis in neutrophils, a terminally differentiated cell which has no use for cyclins in the traditional sense of cell cycle regulation. The first results chapter of this thesis (Chapter 3) aims to establish the effects of R-roscovitine on neutrophil apoptosis and any similarities in action between R-roscovitine and the fungal metabolite gliotoxin. Gliotoxin was chosen as it induces neutrophil apoptosis in a similar time scale to that of R-roscovitine and its mechanism of action is better understood. This could potentially cast insight into the as yet unknown pathways through which R-roscovitine induces neutrophil apoptosis. Another aspect of the investigations in this chapter was to scope the potential suitability for the use of R-roscovitine as a novel treatment for neutrophil dominant inflammatory disorders such as Cystic Fibrosis. This was achieved through examining the effect of R-roscovitine on several pertinent markers of neutrophil activation, and probing the effects of R-roscovitine on macrophage clearance of apoptotic neutrophils. The second results chapter (Chapter 4) aims to investigate potential mechanisms of action for R-roscovitine. These include the following pathway and survival proteins which are known to play pivotal roles in the delicate regulation of neutrophil apoptosis: NF- $\kappa$ B; the NF- $\kappa$ B regulated survival protein XIAP; and the survival protein Mcl-1 which is not directly regulated by NF- $\kappa$ B activation.

## **Chapter 2**

### **Materials and Methods**

#### **2.1 Reagents**

All reagents, unless otherwise stated, were obtained from Sigma-Aldrich (Dorset). Iscove's Modified Dulbeccos Medium (IMDM) and Hanks balanced salt solution were purchased from PAA. Dextran T-500 was obtained from G.E. Healthcare, as was Percoll<sup>TM</sup>. Isotonic saline was purchased from Baxter U.K and Diff-Quik<sup>TM</sup> was purchased from Dade Behring, Germany. R-roscovitine (R)-2-[[9-(1-methylethyl)-6-[(phenylmethyl)amino]-9H-purin-2-yl]amino]-1-butanol was obtained from A.G.Scientific, PVDF was purchased from Immobilon-P, Millipore, Kodak Biomax light film was obtained from Kodak USA and Enhanced Chemiluminescence (ECL) was purchased from Amersham Biosciences. Annexin V-fluos (FITC) was purchased from Roche. Recombinant human TNF- $\alpha$ , GM-CSF and IL-8 were all purchased from R&D Systems whilst LTB<sub>4</sub> was from Merck and Dexamethasone was from Organon. LPS (E.Coli serotype 0127:B8), C5a and budesonide were from Sigma-Aldrich as were PAF, gliotoxin and dbcAMP. Mcl-1 primers were from MGW Oligo Synthesis. DIFF- QUIK<sup>TM</sup> was from Gamidor and the Limulus Amoebocyte Lysate Chromogenic Endpoint Assay was from Hycult. MG-132 (N-cbz-Leu-Leu-leucinal) was from Biomol, Affinity Research Products and zVAD-fmk (benzylocarbonyl-Val-Ala-Aspfluoromethylketone) was from Bachem Ltd.

#### **2.2 Antibodies**

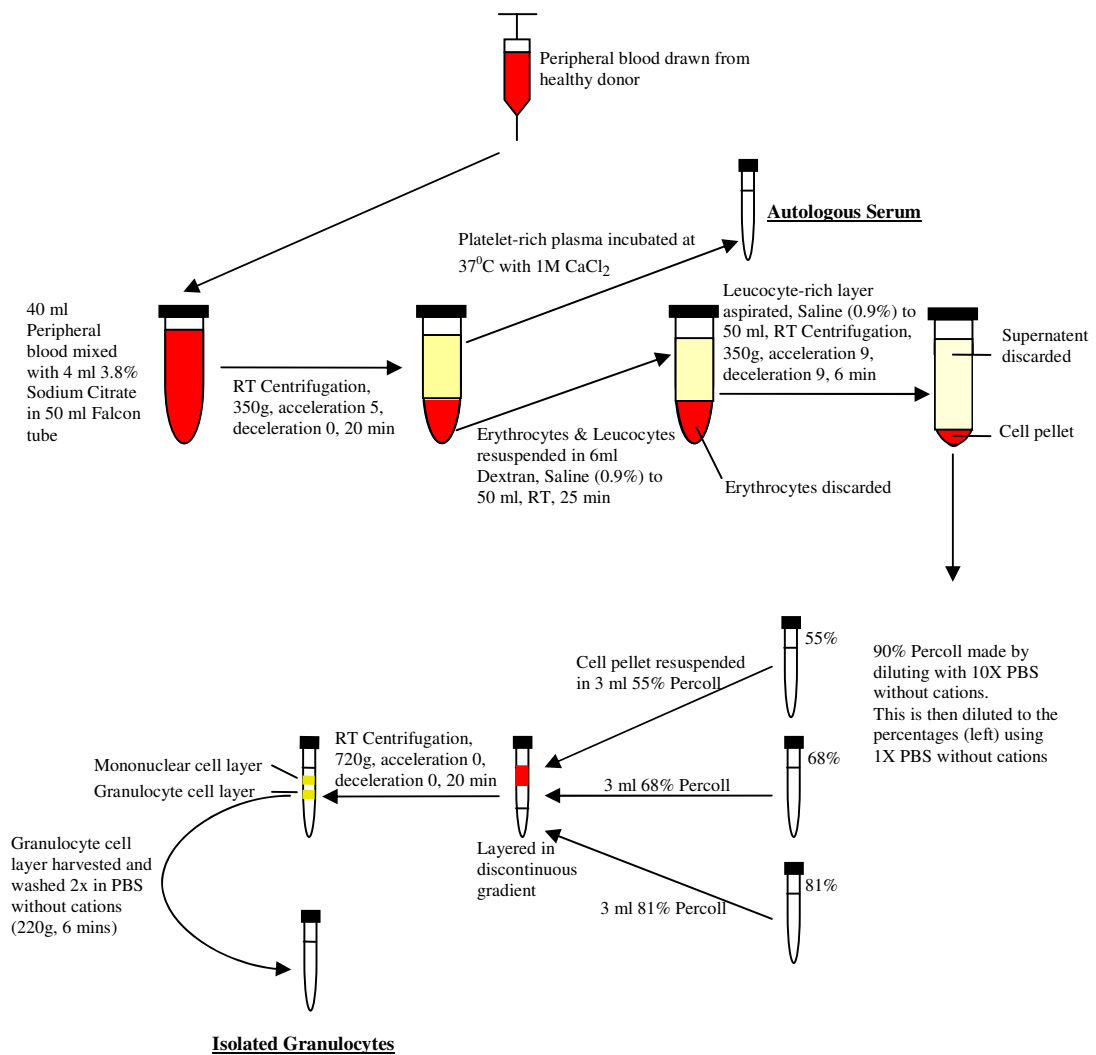
All antibodies were purchased from Santa Cruz unless otherwise stated. Monoclonal anti  $\beta$ -actin was purchased from Sigma-Aldrich, anti-human I $\kappa$ B $\alpha$  was obtained from Abcam and anti-human X-IAP was purchased from R and D Systems. Anti-CD62L-PE and anti-CD11b-APC were obtained from BD Bioscience. Anti-Caspase-3 was purchased from Cell Signaling Technologies and Goat anti-mouse and rabbit HRP-conjugated antibodies were purchased from Dako.

### **2.3 Isolation of mononuclear and polymorphonuclear leucocytes from whole blood.**

Aliquots of 40 ml of human peripheral blood were collected from healthy volunteers, according to Lothian Research Ethics Committee approval #08/S1103/38 or #1702/95/4/72, into 50 ml Falcon tubes containing 4 ml of 3.8 % sodium citrate (Phoenix Pharmaceuticals Ltd., Gloucester, UK). Each Falcon tube was then gently inverted 3 times to ensure that the blood was adequately mixed with the anti-coagulant. The blood was then centrifuged at room temperature at 350g, acceleration 5 and deceleration 0 for 20 min to separate out the platelet rich plasma. The top layer consisting of the platelet rich plasma was aspirated and converted into autologous serum by adding 10 ml to a glass tube containing 200  $\mu$ l of 1 M calcium chloride. This tube was mixed by gently inverting and incubated in a 37 °C water bath for approximately 1.5 h, enabling the platelets to aggregate.

To the bottom layer containing the erythrocytes and leucocytes, 2.5 ml of 6 % (w/v) Dextran T500 per 10 ml of haematocrit was added into each of the tubes. The volume was then made up to 50 ml with 0.9% saline which had been incubated to 37 °C, the Falcon tubes inverted 3 times and the lids loosened. Any excess blood that had collected within the lid following mixing was aspirated and any bubbles removed. The lid was rested back on the tube and incubated at room temperature for 25 min or until the erythrocytes had sedimented to the 25 ml mark. Whilst the erythrocyte rich layer was sedimenting, Percoll™ gradients were prepared for the separation of the mononuclear cells and the granulocytes. 100 % Percoll™ was diluted to 90 % using 10 X Dulbecco's Phosphate Buffered Saline (PBS) without calcium or magnesium cations. Percoll™ densities of 81 %, 68 % and 55 % were prepared by adding 8.1 ml 90 % Percoll™ to 1.9 ml 1 X PBS (without  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ), 6.8 ml 90 % Percoll™ to 3.2 ml 1 X PBS (without  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ), and 5.5 ml 90 % Percoll™ to 4.5 ml 1 X PBS (without  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) respectively. At this point the leukocyte rich upper layer was gently aspirated off into fresh Falcon tubes and the volume made up to 50 ml with saline. The tubes were then centrifuged (350g, acceleration 9, deceleration 9, 6 min, RT) to form a cell pellet. Whilst the tubes were being centrifuged, 3 ml of the 68 % Percoll™ density was carefully layered on top of the 81 % Percoll™ density in a 15 ml Falcon tube. After centrifugation, the supernatant from the 50 ml Falcon tubes was

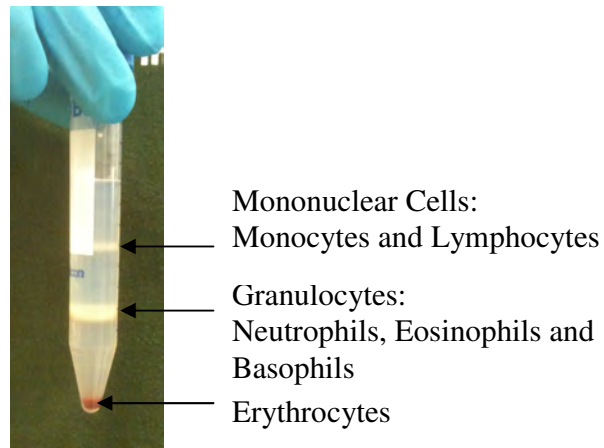
carefully discarded and the pellets resuspended in 3 ml of 55 % Percoll<sup>TM</sup>, this was then carefully added as the final layer to the gradient. Gradients were then centrifuged at 720g, acceleration 0 and deceleration 0 for 20 min at room temperature. The mononuclear cells were harvested from the 68 % / 55 % interface and the granulocytes harvested from the 68% /81 % interface. The purified cells were then washed twice with 50 ml of PBS (without  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$ ) and counted using a haemocytometer. A single drop of the washed granulocytes was placed on the haemocytometer slide and visualised under a light microscope, the number of neutrophils present in 25 squares were counted (cell activation by shape change could also be observed at the same time). The number obtained was equal to the number of neutrophils per 0.1  $\mu\text{l}$  PBS, to calculate the number of neutrophils in 1 ml this was multiplied by 10,000.



**Figure 2.1 Diagrammatic representation of the isolation of human granulocytes from whole blood**

*Peripheral blood is drawn from a healthy volunteer is separated by centrifugation allowing removal of the platelet rich plasma from the erythrocytes and leucocytes. Dextran sedimentation is then used to enable aspiration of the leucocyte rich upper layer. The leucocyte rich upper layer once exposed to centrifugal force produces a leucocyte rich pellet, which is separated out into mononuclear cells and granulocyte cells by Percoll density gradient separation.*



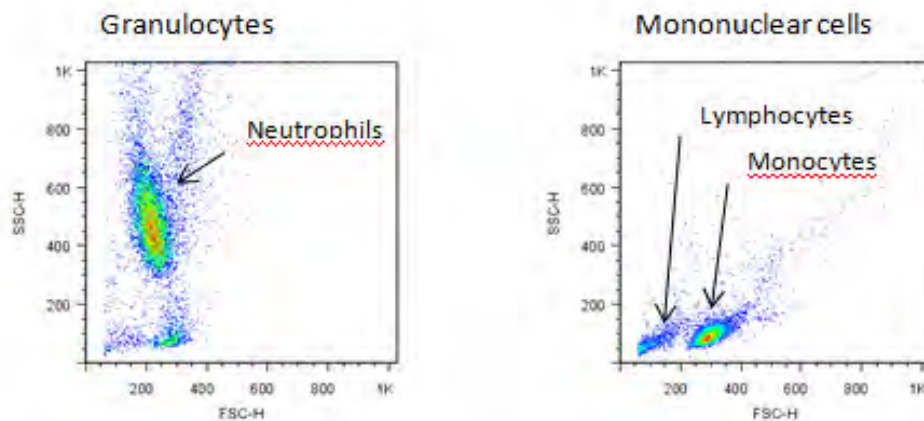


**Figure 2.2 Photograph demonstrating separation of freshly isolated human blood cells by Percoll™ density gradient**

*Two distinct bands and a pellet are produced; the smaller top band contains the mononuclear cells comprised of monocytes and lymphocytes, the larger lower band contains the granulocytes – neutrophils, eosinophils and basophils, and the pellet is comprised of the erythrocytes.*

#### **2.4 Assessment of Neutrophil Purity and Viability**

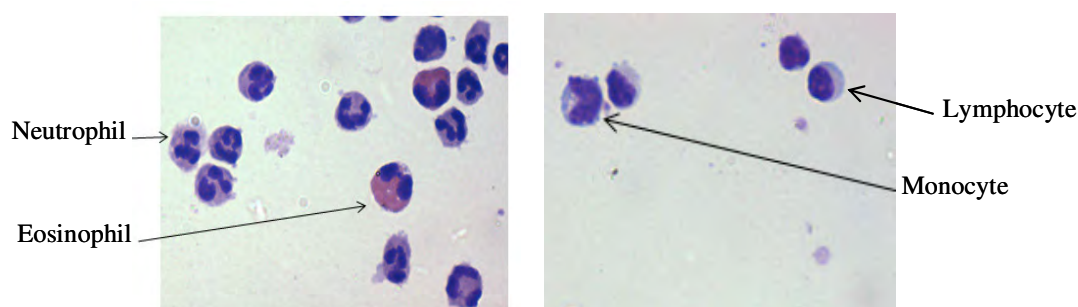
The purity of the preparations was checked using flow cytometry as follows; the percentage of neutrophils was assessed by labeling the PMN fraction with anti-human CD16 RPE detected by the FL-2 detector on the flow-cytometer.



**Figure 2.3 Flow cytometric dot plots of freshly isolated human granulocytes and monocytes demonstrating the cell populations.**

*Flow cytometry allows the purity of the preparations to be assessed. As can be seen in the flow plot on the left the granulocyte fraction is mainly comprised of neutrophils. The flow plot on the right (mononuclear cells) shows two distinct populations: lymphocytes and monocytes.*

Cytocentrifuged cells stained with DIFF QUIK™ were used to calculate the percentage of eosinophils so that only preparations in which the granulocyte layer comprised of < 5% eosinophils and > 95% neutrophils were used and from this point on will be referred to as neutrophils. The typical yield using this method of cell isolation was approximately 200 – 600 million polymorphonuclear cells per 160 ml of blood.



**Figure 2.4 Freshly Isolated Diff-Quik™ stained granulocytes and mononuclear cells (x1000 magnification)**

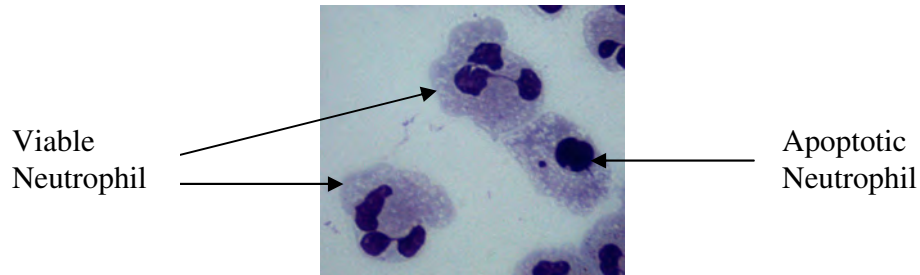
*Neutrophils are identified by the light pink stain of their cytoplasm, as they are less permeable to the stain Eosin than eosinophils, in which Eosin results in the appearance of a dark pink cytoplasm. Neutrophils also have a distinctive multi lobed nucleus as opposed to the eosinophils bi-lobed nucleus. Basophils are rarely seen as they comprise < 1 % of the circulating leucocytes. The monocytes are identified by their larger size and cytoplasm and by the irregular shape of their nucleus. In comparison lymphocytes are usually smaller, possess a smaller cytoplasm and have a more regularly shaped nucleus.*

## **2.5 Methods of assessing neutrophil apoptosis**

### **2.5.1 Cell Morphology**

The morphology of neutrophils was examined by cytocentrifugation (100  $\mu$ l of  $5 \times 10^6$  cells/ml at 300 rpm for 3 min). The cells were then fixed for 1 min in 100 % methanol and stained using DIFF QUICK™ as follows: 1 min incubation in the Eosin stain followed by a 1 min incubation in the Haematoxylin stain. Slides were then rinsed with water and left to dry prior to mounting using DPX. Cells were viewed under oil immersion light microscopy x 1000 magnification. Figure 2.4 shows the morphological changes that take place in the neutrophil during apoptosis; there is a reduction in granulocyte volume, the chromatin condenses and the DNA is cleaved internucleosomally resulting in loss of the multi lobed appearance of the nucleus. The cellular membrane remains intact during apoptosis

preventing the unregulated release of the neutrophils histotoxic contents into the extra cellular milieu.



**Figure 2.5 Viable and apoptotic Diff-Quik™ stained human neutrophils 20 h (x 1000 magnification)**

*The photograph above depicts neutrophils which have been aged for 20 h. Apoptotic neutrophils are identified by the loss of the multi lobed appearance of their nucleus.*

### **2.5.2 Annexin V / Propidium Iodide (PI) Staining**

This method is used to quantify apoptosis as recombinant human Annexin V (labelled with Fluorescein isothiocyanate (FITC)) binds to phospholipids and has a strong affinity for phosphatidylserine (PS). On viable cells PS is located on the inner monolayer surface of the plasma membrane. However, when a cell undergoes apoptosis, PS becomes exposed on the outer leaflet of plasma membranes allowing the FITC labelled Annexin V to bind. The FITC label can be detected using flow cytometry and therefore apoptosis can be assessed. Propidium iodide (PI) is a fluorescent molecule which intercalates into DNA upon loss of membrane integrity and therefore is used as it is a marker for necrosis. Annexin V was diluted 1/500 in binding buffer (Hanks balanced salt solution +) and 280 µl added to 20 µl of cells ( $5 \times 10^6$  cells/ml). Samples were then incubated on ice at 4 °C for 10 – 15 min. Then just prior to running on the flow cytometer 1 µl of PI (1 mg/ml) was added per sample.

## **2.6 Western Blotting for Mcl-1, X-IAP and I $\kappa$ B $\alpha$**

Western Blotting was used to investigate the anti-apoptotic proteins Mcl-1 and X-IAP at the translational level. It was also used to assess the presence of I $\kappa$ B $\alpha$ , which inhibits the NF $\kappa$ B heterodimer holding it in the cytoplasm and prevents the translocation of the transcription factor into the nucleus where it can initiate the synthesis of anti-apoptotic proteins such as X-IAP. Cells at a concentration of  $5 \times 10^6$  /ml per condition were incubated at 37 °C on a shaking heat block, incubation time varied depending upon the focus of the investigation (please see figure legends for incubation times, reagents and concentrations). Cells were then lysed as follows.

### **2.6.1 Neutrophil lysis**

Cells were pelleted using a bench top centrifuge at 6000g for 30 s. The pellet was resuspended in 180  $\mu$ l of whole cell lysis buffer [500  $\mu$ l Sigma protease cocktail P8340 – 1/50 dilution in 1 x TBS, plus 30  $\mu$ l 1 x TBS plus 20  $\mu$ l AEBSF (100 mg to 834  $\mu$ l H<sub>2</sub>O), 20  $\mu$ l Aprotinin (5 mg in 5 ml H<sub>2</sub>O), 20  $\mu$ l Leupeptin (50 mg in 5 ml H<sub>2</sub>O), 40  $\mu$ l Pepstatin A (5 mg in 5 ml methanol), 20  $\mu$ l Sodium Vanadate (0.5 g in 2.72 ml H<sub>2</sub>O pH 10), 20  $\mu$ l Benzamidine (0.1 g in 1.277 ml H<sub>2</sub>O), 20  $\mu$ l Levamisole (1 g in 2.076 ml) and 60  $\mu$ l  $\beta$ -Glycerophosphate (1g in 1389  $\mu$ l H<sub>2</sub>O)]. Such an extensive array of protein inhibitors was required to overcome the numerous proteases contained within the neutrophils which form part of its arsenal against invading pathogens. If these proteases were not adequately inhibited cleavage of proteins could occur during the lysis process. Samples were then incubated on ice for 10 min. Following this, 20  $\mu$ l of 10 % NP-40 was added and samples quickly vortexed before being incubated on ice for a further 10 min. The samples were then centrifuged at 8000g in a bench top centrifuge at 4 °C for 20 min. The supernatant was removed and transferred to a fresh Eppendorf, 100  $\mu$ l of 4 x sample buffer (30 % glycerol; 10 %  $\beta$ -mercaptoethanol; 8 % SDS; 0.25 M Tris HCl pH 6.8, 0.02 % bromophenol blue) was then added and the samples boiled on a 95 °C heat block for 5 min. Samples can be stored at -20 °C for a maximum of 2 days.

### **2.6.2 Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE)**

Samples were loaded into wells on a 4.5 % acrylamide stacking gel; the stacking gel allows the proteins to become concentrated at the dye front. A 12 % separating gel was also used as a higher percentage of acrylamide results in the proteins migrating at a slower rate through the gel, this enables lower molecular weight proteins to undergo adequate separation whilst still remaining on the gel.

#### **4.5% Stacking gel solution**

For one gel:

Distilled water	4.3 ml
Stacking Buffer (0.5 M Tris pH 6.8)	2.5 ml
Acrylamide/Bisacrylamide Mix	1.5 ml
20 % SDS	100 µl
10 % APS	60 µl
TEMED	30 µl

#### **12% Separating gel solution**

For one gel:

Distilled water	3.4 ml
Separating Buffer (1.5 M Tris pH 8.8)	2.4 ml
Acrylamide/Bisacrylamide Mix	4 ml
20 % SDS	50 µl
10 % APS	40 µl
TEMED	20 µl

### **2.6.3 Western Blotting**

Gels were run at 70 V (stacking gel) and 150 V (separating gel). The gels were then transferred at either 60 V for 1 h at room temperature (or 150 V for 30 min in a cold room) onto polyvinylidene difluoride (PVDF) membrane. The membranes were blocked for 1 h on a shaking plate at room temperature in 5 % Marvel / TBS / 0.1 % Tween-20) which blocks non specific binding sites. Blots were washed 3 x 5 min in TBS / 0.1% Tween-20 and the primary antibody (diluted in 5 % Marvel / TBS / 0.1 % Tween-20) added and incubated for 1 h on a shaking plate (Mcl-1 and X-IAP antibodies) (or overnight at 4 °C for the IκBα antibody). Subsequently blots were washed 3 x 5 min in TBS / 0.1% Tween-20 and the secondary antibody (HRP labelled), which was diluted 1 / 2500 in 5 % Marvel / TBS / 0.1 % Tween-20 was added and incubated for 1 h at room temperature on a shaking plate. Blots are then washed 3 x 5 min in TBS / 0.1% Tween-20 and bands visualized using electrochemiluminescence (ECL) reagent which results in chemiluminescence at sites where the HRP labelled protein is bound to the membrane antigens due to peroxidase catalysed oxidation of luminol.

## **2.7 Immunohistochemistry and Confocal analysis.**

### **2.7.1 Annexin V Binding and Propidium Iodide Staining**

Freshly isolated neutrophils at  $2.5 \times 10^6$  /ml were aliquoted at 100 µl into a flat bottomed flexiwell plate, centrifuged at 400g, 4 °C for 4 min and the supernatants removed. Cell pellets were resuspended and fixed for 20 min in 3 % PFA. After washing the cells were resuspended in 5 µl of goat serum (to block non specific binding sites) and blocked for 1 h. Cells were then centrifuged (as before), the supernatant removed, cells resuspended and incubated with 50 µl of the p65 primary antibody diluted 1:50 and 50 µl of PI (2 µl in 1 ml) for 1 h. Once washed the cells were incubated with 50 µl of alexa fluora green frag 1:500 for 1 h, washed and resuspended to 150 µl. 100 µl were removed, cytocentrifuged onto slides and visualized on confocal.

## **2.7.2 Phagocytosis assay for apoptotic neutrophils.**

### **2.7.2.1 Cell isolation and culture**

Freshly isolated monocytes at  $4 \times 10^6$  /ml in IMDM were plated at 500  $\mu$ l/well and incubated for 1 h at 37 °C in a 5 % CO<sub>2</sub> environment. The cells were then washed and the medium replaced with IMDM + 10% autologous serum (for reagent concentrations see figure legends) and cultured into macrophages over 5-7 days via a process of selective adherence (where the macrophages adhere to the plate allowing the other cells to be washed off).

### **2.7.2.2 Aging neutrophils**

Freshly isolated neutrophils were resuspended at  $20 \times 10^6$  /ml in IMDM and were labeled for 15 min at 37 °C, 5 % CO<sub>2</sub> using 1  $\mu$ l CM green /  $10 \times 10^6$  cells/ml. Following this the cells were centrifuged at 200g for 5 min at room temperature and the supernatant removed. They were resuspended at  $4 \times 10^6$  /ml in IMDM plus 10% autologous serum and then incubated at 37 °C, 5 % CO<sub>2</sub> for 20 h.

### **2.7.2.3 Phagocytosis**

The greened neutrophils were centrifuged at 200g for 5 min at room temperature and then resuspended in IMDM at  $4 \times 10^6$  /ml. 500  $\mu$ l of neutrophils per well were added to the washed macrophages and incubated for 1 h at 37 °C, 5 % CO<sub>2</sub>. Following this the medium was removed and the macrophages detached by incubating with 0.25 % trypsin / 1 mM EDTA at 37 °C for 15 min prior to a 15 min incubation at 4 °C. The cells were then resuspended via pipetting then analysed by flow cytometry.



### **2.7.3 Assessment of CD62L, CD11b and Shape Change**

Neutrophils were resuspended at  $5 \times 10^6$ /ml in PBS + cations and treated with reagents as detailed in the appropriate figure legend. Treatments took place on a 37 °C shaking heat block. 50 µl of cells were then added to a facs tube containing 150 µl ice cold PBS without cations and treated with 2 µl of either the control APC/PE antibodies or with antibodies for CD62L, CD11b for 30 min prior to running the samples on the flow cytometer.

### **2.8 X-IAP HIV –tat**

The X-IAP HIV-tat protein was a kind gift from Dr Brigitte Onteniente.

The primers 5-VGGG CTC GAG ATG ACT TTT AAC AGT TTT GAA GG-3V(sense) and 5-VGGG GAA TTC TTA AAA CAT AAA AAT TTT TTT GCT TG-3V(antisense) were used to isolate the cDNA reading frame of rat X-IAP by PCR. The purified fragments were then cloned into a pPTD-HA vector at the XhoI/EcoRI sites.

#### **Final protein encoded:**

6-histidine residues tag, PTD (YGRKKRRQRRR), hemagglutinin (HA) tag (YPYDVPDVA), and X-IAP

The E.coli strain BL21(DE3) pLysS was used to express the plasmids and 500 AM isopropyl 1-thioh-d-galactoside was used to induce protein production, these were then extracted in 8 M urea HEPES buffer and purified by a Ni-NTA superflow agarose column. A Sephadex G-25M column was used to remove salt by gel filtration, collecting the proteins in a solution containing PBS.

### **2.8.1 Effects of X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 18h.**

Freshly isolated Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum in either with IMDM + 10 % autologous serum (control), 1  $\mu$ M X-IAP-tat, 20  $\mu$ M R-roscovitine or a combination of 1  $\mu$ M X-IAP-tat + 20  $\mu$ M R-roscovitine (XR) and incubated at 37 °C for 18 h before staining with annexin-V and PI, levels of which were assessed by flow cytometry (see Annexin V Staining and Propidium Iodide Binding).

### **2.8.2 Effects of X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 4h.**

Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum as follows. Control/Control in the absence of any agents for 4 h. Control/R-roscovitine in the absence of any agents for 1 h and then 20  $\mu$ M R-roscovitine added for 3 h. LPS/Control treated with 100 ng/ml LPS for 4 h with media added after 1 h for the remaining 3 h. LPS/R-roscovitine treated with 100 ng/ml LPS for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. X-IAP/Control treated with 1  $\mu$ M X-IAP-tat for 4 h with media added after 1 h for the remaining 3 h. X-IAP/R-roscovitine treated with 1  $\mu$ M X-IAP-TAT for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. Samples were then stained with annexin-V and PI levels of which were assessed by flow cytometry

### **2.8.3 X-IAP-tat construct effect on neutrophil shedding of CD62L and Shape Change**

Neutrophils were resuspended at  $5 \times 10^6$  /ml in PBS + cations. Half of the samples were treated with media that contained 10 % autologous serum and half were treated without serum. Within each of these two conditions one sample was treated without any additional reagents (control) one was treated with 100 ng/ml LPS and one with 1  $\mu$ M X-IAP-tat for 30 min prior to incubation with anti-human anti-CD62L for 30 min. Samples were then analyzed using flow cytometry.

## **2.9 Limulus Amoebocyte Lysate Chromogenic Endpoint Assay (LAL Assay)**

As the X-IAP-tat construct was produced in a bacterial vector it is important to check that the protein produced has not been contaminated with the bacterial endotoxin LPS, as LPS has a lifespan prolonging effect on neutrophils.

The kit used was purchased from Hycult, the reconstituted standard was diluted with endotoxin free water to give a concentration of 30 EU/ml which was vortexed for 30 s. To produce the readings for the standard curve 16 wells of the plate were filled with 50 µl of endotoxin free water. 25 µl of the 30 EU/ml standard was added to well 1 and thoroughly pipetted to mix, 25 µl was then removed from well 1 and added to well 2 (diluting 1:3), this process was repeated up to well 7. 25 µl was discarded from well 7 and well 8 became a control value from which the standard dilution process was repeated for wells 9-16.

Duplicate 50 µl X-IAP-tat samples (which had been diluted 1:10 with endotoxin free water) were pipetted into their assigned wells. To each well 50 µl of reconstituted LAL reagent was added (to sample controls 50 µl of endotoxin free water was added instead). The plate was covered and incubated at room temperature for 30 min until the standard conditions produced a clear colour formation. The tray was then gently tapped to eliminate air bubbles and mix the samples and placed in a spectrophotometer which measured the absorbance of the samples at 450 nm. The OD value of the control was subtracted from the OD value of the samples and the standard curve produced from the standard conditions. This was used to determine the endotoxin concentration in the X-IAP-tat sample.

## **2.10 RT-PCR for Mcl-1**

Freshly isolated neutrophils ( $25 \times 10^6$ /sample) were pelleted and resuspended in 1 ml Trizol. They were then incubated at room temperature for 5 min, following which 0.2 ml of chloroform was added and the tubes shaken vigorously for 15 s, they were then incubated at room temperature for 2-3 min. After this the samples were centrifuged at 12000g for 15 min at 4 °C, this results in separation into an RNA rich upper fraction, a protein interphase

and a lower fraction of protein and DNA. The upper fraction was removed into a clean tube and 0.5 ml of isopropanol added, they were then incubated on ice for 20 min and then centrifuged at 12000g for 10 min at 4 °C. At this point a pellet of RNA can be seen and the supernatant was removed. The pellet was then washed with 75 % ethanol after which it was vortexed and centrifuged at 7500g at 4 °C for 5 min. The RNA pellet was then air dried for 10 min and then the pellet dissolved in 20 µl of RNase free water and incubated at 55 °C for 10 min after which the sample could be stored at -70 °C.

#### **2.10.1 DNase treatment of RNA samples**

0.1 volume of RNA was added to 10x DNase I buffer, to this 1µl of DNase I enzyme was added. This was mixed gently and incubated for 20-30 min at 37 °C. Then 5 µl of DNase inactivation buffer was added and the samples incubated at 37 °C for 2 min. Samples were centrifuged at 8000g for 1 min and the supernatant removed into a fresh tube and stored at -80 °C. This was performed to remove any contaminating DNA from the samples.

#### **2.10.2 Making cDNA from RNA**

1 µl of oligo DT was added to 2 µg of RNA (13.3 µl of RNA) and to this 4 µl of first strand buffer, 2 µl of DTT (0.1 M), 0.5 µl of 20 mM dNTP and 0.5 µl of RNase inhibitor mix were added. This was incubated at 42 °C for 2 min, then 1 µl of superscript enzyme was added and samples heated at 42 °C for 50 min and then at 70 °C for 15 min.

#### **2.10.3 RT-PCR**

19 µl of water was added to 0.5 µl of 10 x buffer, 0.5 µl of dNTP 10 mM, 0.75 µl of 50 mM MgCl<sub>2</sub>, 0.5µl of forward primer, 0.5 µl of reverse prime, 1 µl of cDNA and 0.25 µl of Taq and run using the following PCR program:

Heated lid 105 °C  
Heated lid before program on  
Pause before program off  
Initial denaturation 5 min at 94 °C  
Hot start disabled  
Number of cycles 35  
Seg max c/s 30 s, 94 °C  
Seg max c/s 30 s, 56 °C  
Seg max c/s 30 s, 72 °C  
Final hold 4 °C  
Products were stored at -4 °C

#### **2.10.4 PCR primers**

GAPDH:	Forward 5'-TGCCTCCTGCACCACCAAGTG-3'
(control)	Reverse 5'-AATGCCAGCCCCAGCGTCAAAG-3' (450bp)

Mcl-1:	Forward 5'-GAGGAGGAGGAGGACGAC-3'
	Reverse 5'-CCAGCTCCTACTCCAGCAAC-3' (530bp)

#### **2.10.5 1% Agarose Gel and Running Buffer**

0.6 g of agarose was added to 60 ml 0.5 TBE which was then heated and once cooled slightly 2.5 ml of ethidium bromide added. This was then poured into a mould, a well comb added and left to set. The running buffer was made as follows:

Stock 5x TBE  
1960 ml distilled water  
40 ml, 0.5 M EDTA, pH 8  
108 g Tris  
55 g Boric acid

Samples were ran on the 1 % (w/v) agarose gel and visualised by ethidium bromide staining under UV light.

### **2.11 Statistical Analysis**

Experiments were analysed by analysis of variance (ANOVA) and Student-Newman-Keul multiple comparison post hoc test (InStat software). Data are expressed as the mean  $\pm$  s.e.m. and values of  $P < 0.05$  were considered significant.  $P < 0.05 = *$ ,  $P < 0.01 = **$ ,  $P < 0.001 = ***$ .

### **2.12 Densitometry**

Densitometry was performed on western blots using Image J software.

## **Chapter 3**

### **Regulation of neutrophil apoptosis by the CDK inhibitor R-roscovitine and its effects on subsequent macrophage phagocytosis.**

#### **3.1 Introduction**

The inflammatory response is a highly regulated and intricately linked complex series of biochemical reactions and cellular events which have evolved to defend and repair the host organism against infection and injury and restore tissue homeostasis.

As mentioned in the introduction, the neutrophil is an important cell in the innate immune response helping remove invading pathogens. Functionality of the neutrophil is strongly influenced by the surrounding environment. The presence of agents such as GM-CSF, G-CSF, LPS, C5a, IL-1 $\beta$ , IL-2, IL-8, IL-15, INF- $\gamma$ , TNF- $\alpha$  (incubation periods greater than 12 h), glucocorticoids, cAMP, LTB<sub>4</sub> and hypoxia all have a lifespan prolonging effect on neutrophils, allowing them greater time to remove foreign bodies. However, if IL-4, theophylline or PGD<sub>2</sub> metabolites are encountered neutrophil death is triggered (Riley et al., 2006). Therefore a delicate balance between the signals received from the inflammatory environment and the neutrophil's default pro-apoptotic state exists. Timely death of the neutrophil by apoptosis and subsequent removal by professional phagocytes is vital for the resolution of inflammation as disruption of this can exacerbate chronic inflammatory disorders through dysregulation of cellular function and subsequent tissue damage (Leitch et al., 2009).

##### **3.1.1 CDKs and the neutrophil**

Cyclin-Dependent Kinase Inhibitors (CDKis) such as R-roscovitine are serine/threonine kinases inhibitors and are currently in clinical trials for use as novel chemotherapeutic agents, inducing apoptosis in actively proliferating cancerous cells through the Bcl-2 family members and activation of the caspases (Hui et al., 2009). Oral R-roscovitine is currently in

phase II clinical trials to evaluate its anti-tumour activity on advanced solid tumours (in skin and peripheral blood mononuclear cells) when administered sequentially with oral sapacitabine (a drug which is under investigation for its ability to interfere with DNA synthesis). The study which commenced in February 2009 is currently recruiting participants and the trial is due for completion in September 2012.

A further phase I clinical trial investigating the maximum tolerated dose of R-roscovitine in combination with liposomal doxorubicin is also being established for patients with metastatic triple negative breast cancer. The study is scheduled to run from September 2012 until September 2015.

R-roscovitine was investigated as a potential treatment for non-small cell lung cancer although this trial was unfortunately terminated at the phase II stage. However, the CKDi PD0332991 which inhibits CDK4/6 is currently in phase II clinical trials for previously treated, advanced non-small cell lung cancer patients with wildtype retinoblastoma protein and inactive (CDK)N2a and is due for completion in February 2014. The efficacy of PD0332991 is also being tested on recurrent ovarian cancers which demonstrate an Rb-proficiency and low p16 expression (phase II trials due for completion in January 2014); advanced refractory colorectal cancer (phase I study in combination with 5-fluorouracil and oxaliplatin) and in combination with bortezomib for patients with relapsed mantle cell lymphoma. Other CDKs currently being investigated in clinical trials for their anti-cancer abilities are BAY1000394 (in phase I trials for advanced malignancies) and SCH727965 (in phase II trials for multiple myeloma).

The name cyclin-dependent kinase is derived because of their interaction with the cyclins, a family of proteins which control passage between the phases of the cell division cycle. Fascinatingly, although neutrophils are terminally differentiated cells that remain in the G0 phase, therefore would have no use for CDKs in the traditional sense, neutrophils have been found to contain several CDKs including CDK1, CDK2 and CDK5. Contrary to the effect of CDKs on fully proliferated neurons, where they have an anti-apoptotic effect (Monaco and Vallano, 2003), our laboratory has postulated that CDKs present in neutrophils may



have an important role in apoptosis and has discovered that CDKs including R-roscovitine have a powerful ability to induce neutrophil apoptosis (Rossi et al., 2006). Interestingly, it has recently been discovered in our laboratory (Leitch and Rossi, unpublished) that the repertoire of CDKs present in neutrophils is greater than first thought and now includes CDK7 and CDK9, an exciting premiss seen that these two CDKs are particularly important in eukaryotic translation (MacCallum et al., 2005). This finding is also supported by Farahi *et al.* who reported in 2011 that eosinophils contain CDKs 1, 2, 5, 7 and 9 (Farahi et al., 2011).

The mechanism(s) through which CDKs and the different CDKs influence neutrophil apoptosis have yet to be elucidated. Therefore, the purpose of the investigations in this chapter was to further study the apoptosis inducing effect of R-roscovitine on neutrophils. When contemplating this it is important to consider the inflammatory milieu and the survival mediators that the neutrophil may encounter. Therefore in this set of experiments R-roscovitine was used in conjunction with agents commonly found at sites of inflammation. Another focus of this chapter was to examine the effects of R-roscovitine on important markers of neutrophil activation; CD62L and CD11b expression and neutrophil shape change (assessed by flow cytometry). Perturbation of the above could potentially influence recruitment of neutrophils from the circulation to sites of inflammation. Furthermore, I also examined how R-roscovitine interacts with other cells important in the resolution of inflammation, namely the macrophages, and their ability to aid resolution at the inflammatory loci through removal of apoptotic neutrophils.

The effect of R-roscovitine on neutrophil activation and macrophage phagocytosis of apoptotic cells are both extensive areas that, in their own rights, could be the subject of a thesis. However, it was chosen to investigate these areas, at least partially, as a gap in the market exists for a novel anti-inflammatory agent that possesses the ability to induce neutrophil apoptosis in conditions where this is compromised (such as Cystic Fibrosis) without perturbing (and ideally enhancing) the removal of apoptotic cells by the phagocytes.

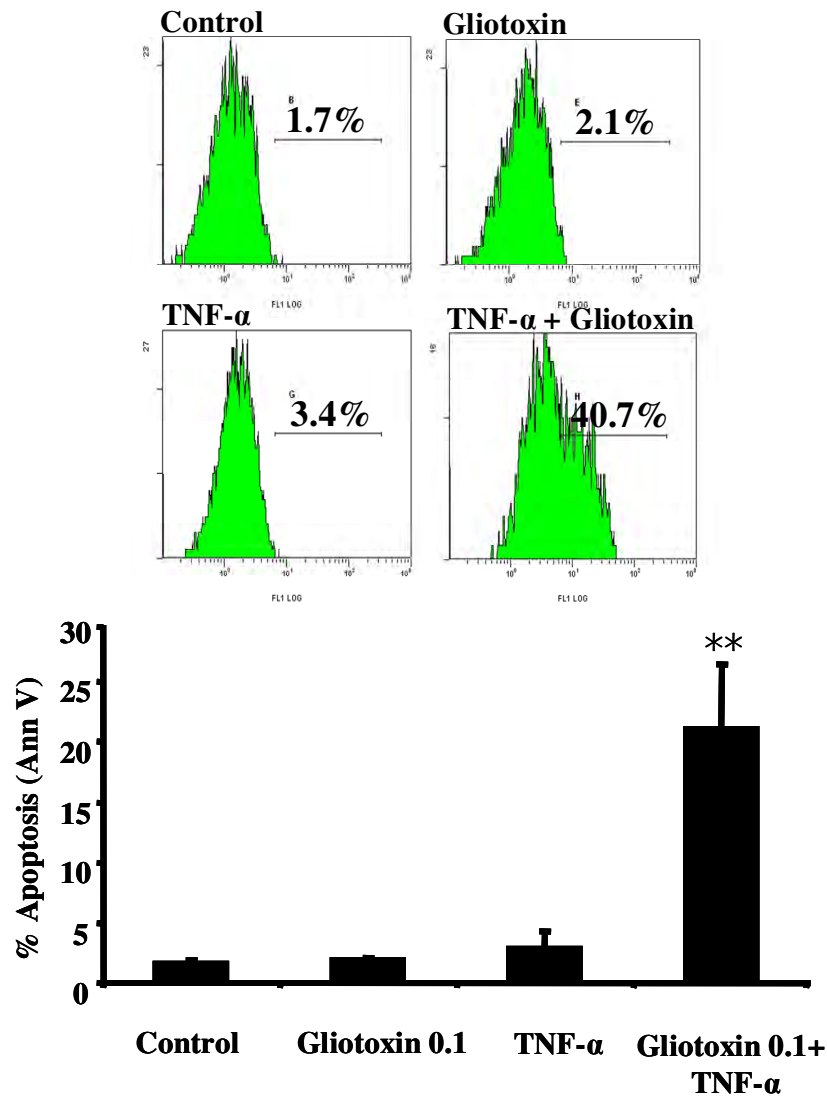
## **3.2 Results**

### **3.2.1 Effect of TNF- $\alpha$ and the NF- $\kappa$ B inhibitor gliotoxin on neutrophil apoptosis at 2 h**

The first sets of experiments were performed to establish parameters and confirm the previously documented effects of a well known inducer of neutrophil apoptosis, gliotoxin. The fungal metabolite, gliotoxin, belongs to the epipolythiodioxoperazine family and is thought to influence neutrophil longevity through a well documented mechanism of NF- $\kappa$ B inhibition to bring about neutrophil apoptosis (Ward et al., 1999a). In order to try and gain some insight into the as yet unidentified mechanism through which R-roscovitine induces neutrophil apoptosis, the effects of gliotoxin were examined. Gliotoxin was chosen as it has been suggested that R-roscovitine can induce apoptosis in cancer cell lines, such as the adenocarcinomic human alveolar basal epithelial cell line A549, through inhibition of I $\kappa$ B-kinase preventing effective phosphorylation of I $\kappa$ B $\alpha$  and subsequently defective suppression of NF- $\kappa$ B (Dey et al., 2007).

In this set of experiments the effects of gliotoxin on neutrophil apoptosis at 2 h and on the inflammatory mediator, TNF- $\alpha$ , known to activate the NF- $\kappa$ B pathway in neutrophils was examined (Ward et al., 1999a). The aim of this was to gain further insight into neutrophil apoptosis resulting from inhibition of NF- $\kappa$ B. This could then be further investigated later in the thesis to see if any similarities were present between this and the effects of R-roscovitine, which may suggest a potential role for NF- $\kappa$ B inhibition in R-roscovitine induced neutrophil apoptosis.

The effects of gliotoxin and the inflammatory cytokine TNF- $\alpha$  on neutrophil apoptosis were examined at 2 h. Investigations were conducted using flow cytometric analysis of Annexin V binding to external phosphatidylserine.



**Figure 3.2.1 Effects of gliotoxin and TNF-α on neutrophil apoptosis at 2 h.**

*Neutrophils ( $5 \times 10^6$  /ml) resuspended in IMDM + penicillin/streptomycin supplemented with 10% autologous serum were incubated for 2 h with either media (control), gliotoxin (0.1  $\mu$ g/ml), TNF-α (10 ng/ml) or gliotoxin (0.1  $\mu$ g/ml) + TNFα (10 ng/ml). Annexin V FITC was added and samples incubated at 4 °C for 10-15 min. PI was added to each sample just prior to reading on the flow cytometer. Data represents mean of 4 separate experiments, each performed in triplicate (\*\*  $P < 0.01$ ).*

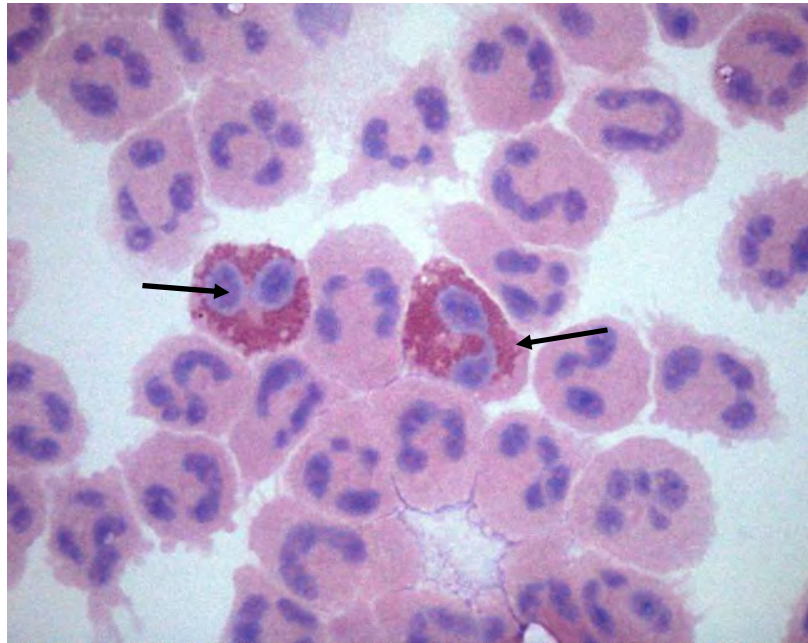
Neutrophils ( $5 \times 10^6$  /ml in 250  $\mu$ l aliquots) were resuspended in IMDM + penicillin/streptomycin supplemented with 10% autologous serum and incubated for 2 h with either media (control), gliotoxin (0.1  $\mu$ g/ml), TNF- $\alpha$  (10 ng/ml) or gliotoxin (0.1  $\mu$ g/ml) + TNF- $\alpha$  (10 ng/ml). Reagent concentrations were chosen from previous work performed in the laboratory.

As previously mentioned TNF- $\alpha$  can have either a pro- or anti-apoptotic affect on neutrophils depending upon the duration of incubation; as can be seen in Figure 3.2.1 TNF- $\alpha$  and gliotoxin alone at 2 h cause little apoptosis (at later time points e.g. 4-8 h significant apoptosis is caused. (Ward et al., 1999a). However, when TNF- $\alpha$  is incubated for 2 h in conjunction with gliotoxin the average percentage apoptosis increased from 1.7 % to 21.1 % ( $P < 0.01$ ) with no necrosis evident. This indicates the importance of the NF- $\kappa$ B pathway in regulation of neutrophil apoptosis (see discussion).

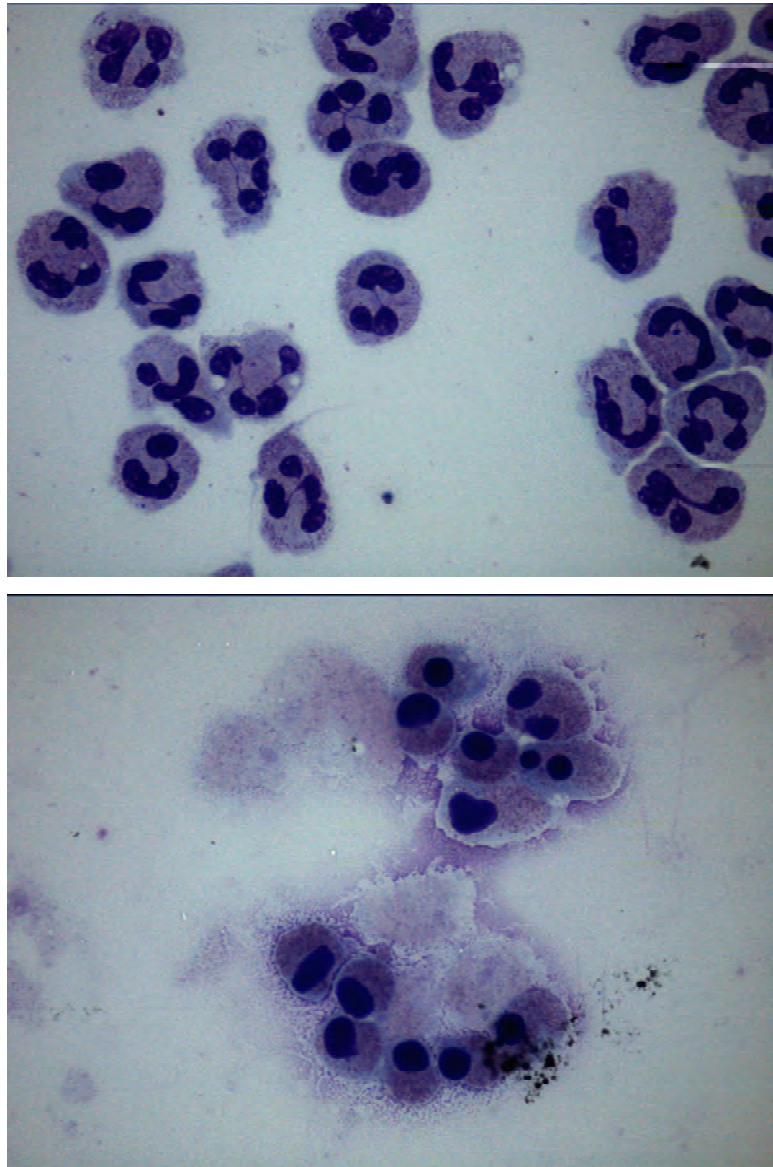
### **3.2.2 Effect of R-roscovitine on neutrophil apoptosis *in vitro* at 4 h.**

Key occurrences in the resolution of inflammation are the timely death of the neutrophil by apoptosis and its removal from the site of infection by professional phagocytes. Many inflammatory conditions can be exacerbated as a result of prolonged survival of activated neutrophils. This results from untimely release of their defensive arsenal of proteolytic enzymes and reactive oxygen species, causing damage to the surrounding tissues (Fox et al., 2010). It is for this reason that novel methods are being sought to stimulate neutrophil apoptosis in systems where desirable rates of apoptosis are compromised. In this set of experiments the ability of the CDK inhibitor R-roscovitine to induce neutrophil apoptosis at early time points was investigated, this is the first step towards identifying potential therapeutic agents for use in the aforementioned capacity.

Figure 3.2.2.1 shows a freshly isolated neutrophil photographed under an oil immersion microscope at a magnification of x1000 visualised by Diff Quik staining.



**Figure 3.2.2.1 Photomicrograph (x1000 magnification) of freshly isolated neutrophils.**  
*Photomicrograph of cytocentrifuge preparations of neutrophils ( $250\ \mu\text{l } 5 \times 10^6 / \text{ml}$ ) freshly isolated from peripheral blood visualised by staining with Eosin and Haematoxylin and viewed under oil immersion microscopy. Two eosinophils (indicated by the arrows) are present in the centre of the picture. These are identified by darker staining of their cytoplasm and their distinctive bi-lobed nucleus.*



**Figure 3.2.2.2 Photomicrograph the effect of R-roscovitine on neutrophil morphology at 4 h.**

*Photomicrograph (x1000 magnification) of neutrophils (250  $\mu$ l  $5 \times 10^6$  /ml). Cells were cultured in either media (A) or 20  $\mu$ M R-roscovitine (B) for 4 h at 37 °C. Cytocentrifuge preparations were made. Slides were stained using Eosin and Haematoxylin and viewed under oil immersion microscopy.*

R-roscovitine was used at a concentration of 20 $\mu$ M as previous studies have shown this concentration to inhibit CDKs specifically (De Azevedo et al., 1997, Meijer et al., 1997, Bach et al., 2005) and experiments in our laboratory supported use of this concentration on neutrophils (Rossi et al., 2006).

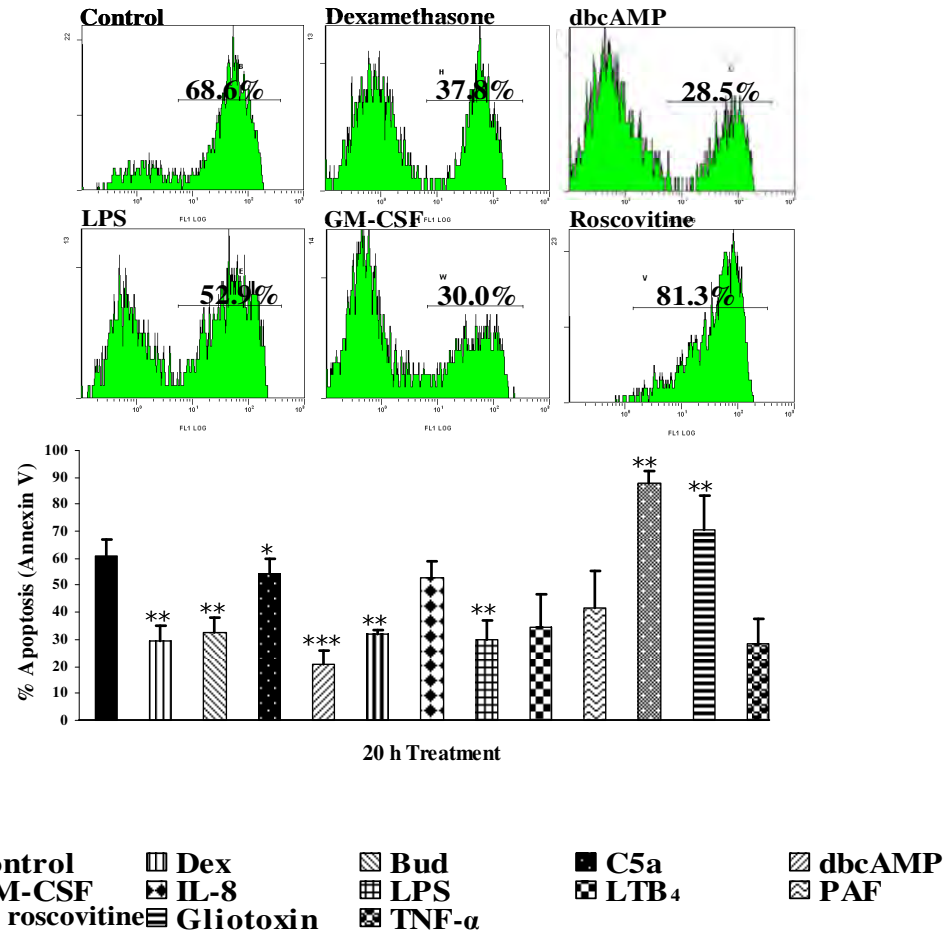
After 4 h incubation at 37 °C on a shaking heat block the appearance of the cells differs little from the freshly isolated neutrophils and the distinctive polymorphic multilobed nucleus and pale cytoplasm are clearly evident (Figure 3.2.2.1 (A)). However, after 4 h incubation with R-roscovitine (20  $\mu$ M) at 37 °C on a shaking heat block (Figure 3.2.2.2 (B)) apoptosis has been induced in a number of neutrophils identified by the condensed circular nucleus, blebbing and a darker cytoplasm. This demonstrates that R-roscovitine has the ability to over-ride the neutrophil's anti-apoptotic mechanisms in as little as 4 h to induce apoptosis.

### **3.2.3 Stimuli affecting neutrophil apoptosis at 20 h**

In order to compare and contrast the effects of R-roscovitine and gliotoxin on neutrophil apoptosis it is important to take into consideration the environment in which neutrophils in an inflammatory disorder reside and critically the survival factors which may be encountered. This is an essential consideration for a novel anti-inflammatory agent targeted to induce neutrophil apoptosis, as it is imperative the agent possess the ability to over-ride powerful survival signals present at sites of inflammation which are responsible for the neutrophils longevity. Lack of this ability would render any agent which induces neutrophil apoptosis useless at the site of inflammatory disorders.

This set of experiments was designed to demonstrate the effects of key pro- and anti-survival factors on neutrophil apoptosis, creating a set of standards with which to compare further experiments investigating the combined administration of apoptosis inducing agents with neutrophil survival agents. This will provide insight into which agents prevail in tipping the delicate balance between death and survival to decide the neutrophil's fate. A

time point of 20 h was chosen as by this time a significant proportion of neutrophils would have entered spontaneous apoptosis.



**Figure 3.2.3 Stimuli effecting neutrophil apoptosis at 20 h.**

*Neutrophils ( $5 \times 10^6$  /ml) resuspended in IMDM + penicillin/streptomycin supplemented with 10% autologous serum were incubated for 20 h, 37 °C, 5 % CO<sub>2</sub> with either media (control), dexamethasone (1  $\mu$ M), budesonide (1  $\mu$ M), C5a (1  $\mu$ M), dbcAMP (0.2mM), GM-CSF (50 ng/ml), IL-8 (100nM), LPS (100ng/ml), leukotriene B4 (1  $\mu$ M), platelet activating factor (1  $\mu$ M), R-roscovitine (20  $\mu$ M), gliotoxin (0.1  $\mu$ g/ml) or TNF- $\alpha$  (10 ng/ml). Annexin V FITC was added to samples which were incubated at 4 °C for 10-15 min. PI was added to each sample just prior to reading on the flow cytometer. Data represents mean of 4 separate experiments, each performed in triplicate. (\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$ ).*



Neutrophils ( $5 \times 10^6$  /ml in 250  $\mu$ l aliquots) were resuspended in IMDM + penicillin/streptomycin supplemented with 10% autologous serum and incubated for 20 h, 37 °C, 5 % CO<sub>2</sub> with either media (control), dexamethasone, budesonide, the complement component C5a, N<sup>6</sup>,2'-O-dibutyryladenosine 3':5' cyclic monophosphate (dbcAMP), GM-CSF, IL-8, LPS, Leukotriene B<sub>4</sub> ( LTB<sub>4</sub>), Platelet-Activating Factor (PAF), R-roscovitine, gliotoxin or TNF- $\alpha$ . Samples were then stained with Annexin V and PI and analysed by flow cytometry.

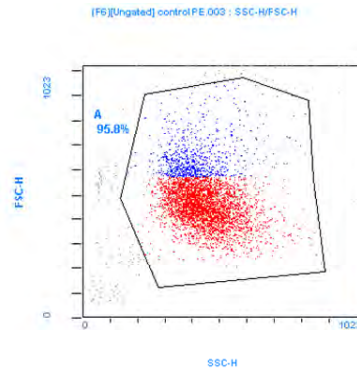
Figure 3.2.3 demonstrates that both glucocorticoids ( $P < 0.01$ ) along with dbcAMP ( $P < 0.005$ ), GM-CSF ( $P < 0.005$ ), LPS ( $P < 0.005$ ), LTB<sub>4</sub>, PAF, TNF- $\alpha$  and to a lesser extent at the concentrations used C5a ( $P < 0.05$ ) and IL-8 all exert a cytoprotective effect on neutrophils, prolonging their lifespan compared to the control, untreated, neutrophils. However, R-roscovitine and gliotoxin both exert a pro-apoptotic affect on neutrophils significantly increasing the rate of neutrophil apoptosis at 20 h compared to the control.

This thesis aims to investigate how a CDKi can induce apoptosis in the terminally differentiated neutrophil.

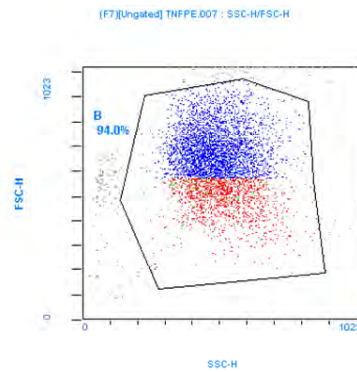
### **3.2.4 Effect of R-roscovitine on markers of neutrophil activation**

In this section we investigated how R-roscovitine and gliotoxin affect the activational state of the neutrophil as this can impact on the longevity of the cell. The effects of R-roscovitine and gliotoxin were examined both independently and in conjunction with prevalent survival factors often encountered at sites of inflammation to ascertain if the pro-apoptotic agents being investigated had any detrimental effect on pertinent markers of neutrophil activation. CD62L shedding, CD11b upregulation and shape change (as assessed by a shift in forward scatter) were investigated by flow cytometry.

**Unstimulated**

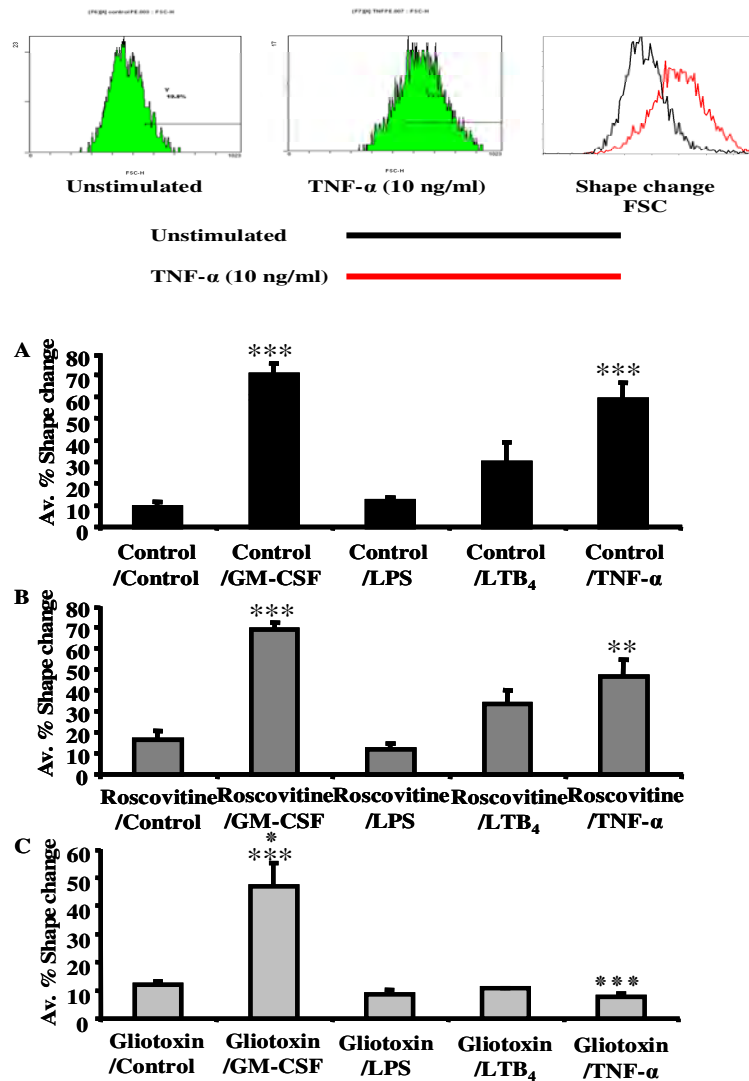


**TNF- $\alpha$   
(10 ng/ml)**



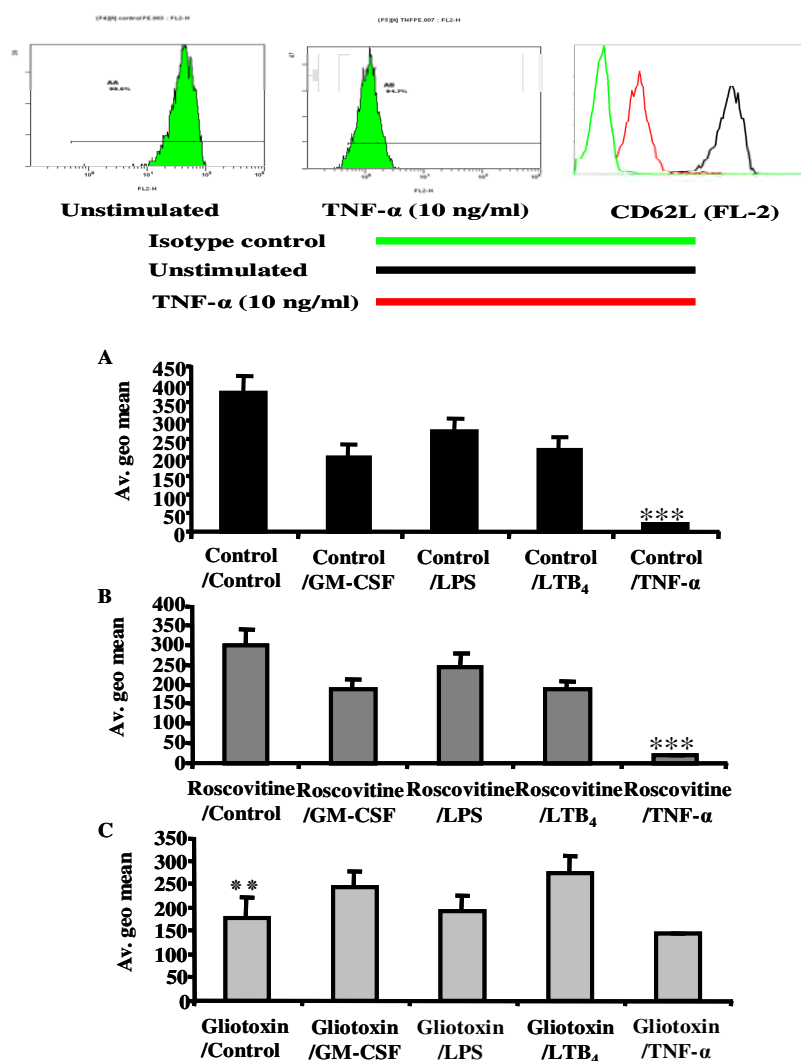
**Figure 3.2.4.1 Flow cytometric scatter plots showing unstimulated neutrophils and neutrophils stimulated with TNF- $\alpha$**

*Neutrophils ( $5 \times 10^6$  /ml) in PBS + cations were incubated either in PBS + cations alone (control) or with TNF- $\alpha$  (10 ng/ml) for 30 min at 37 °C. Samples were stained with control antibodies APC/PE on ice for 30 min and change in forward scatter measured on the BD Calibur flow cytometer. The figure depicts the characteristic shape change that occurs in neutrophils when they are primed by appropriate stimuli. This shape change can be detected as a shift in forward scatter.*



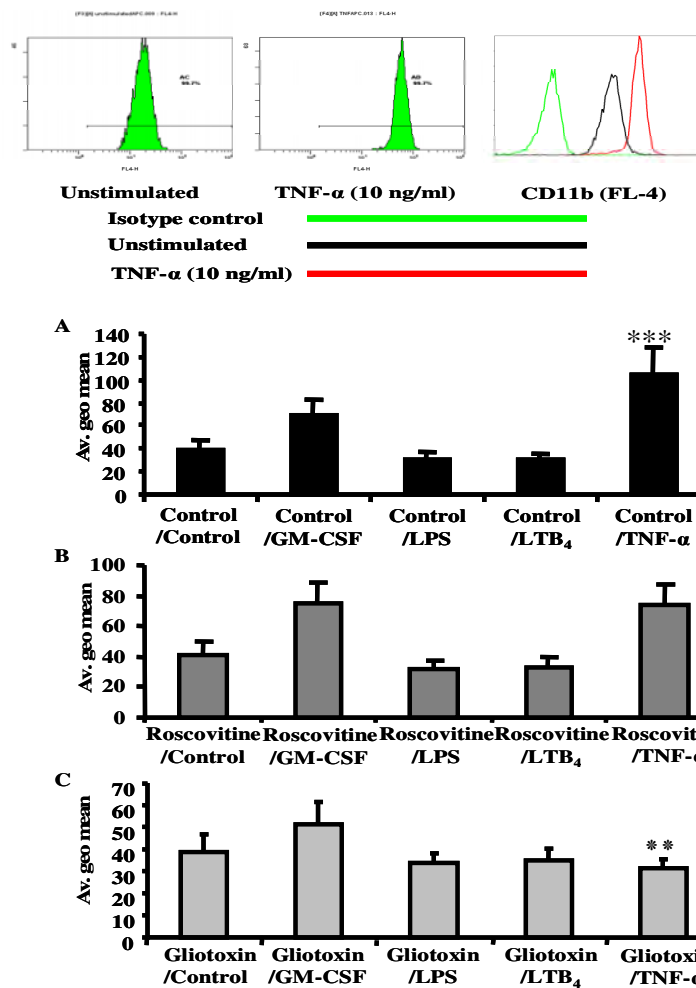
**Figure 3.2.4.2 Effect of R-roscovitine and gliotoxin on neutrophil activation assessed by shape change induced by GM-CSF, LPS, LTB<sub>4</sub> and TNF- $\alpha$**

Neutrophils ( $5 \times 10^6$  /ml) in PBS + cations were pre treated for 30 min on a 37 °C shaking heat block as follows: (A) in PBS + cations alone (control), (B) in 20  $\mu$ M R-roscovitine, (C) in 0.1  $\mu$ g/ml gliotoxin. To (A), (B) and (C) either PBS + (control), GM-CSF (50 ng/ml), LPS (100 ng/ml), LTB<sub>4</sub> (1  $\mu$ M) and TNF- $\alpha$  (10 ng/ml) were added for 30 min at 37 °C. Samples were stained with control antibodies APC/PE on ice for 30 min and change in forward scatter measured on the BD Calibur flow cytometer. Data represents the mean of 4 separate experiments, each performed in triplicate. \* = significant compared to its own control, \*\* = significant when compared to the equivalent condition in the control set of experiments.



**Figure 3.2.4.3 Effect of R-roscovetine and gliotoxin on neutrophil activation assessed by CD62L expression induced by GM-CSF, LPS, LTB<sub>4</sub> and TNF- $\alpha$**

Neutrophils ( $5 \times 10^6$  /ml) in PBS + cations were pre treated for 30 min on a 37 °C shaking heat block as follows: (A) in PBS + cations alone (control), (B) in 20  $\mu$ M R-roscovetine, (C) in 0.1  $\mu$ g/ml gliotoxin. To (A), (B) and (C) either PBS + (control), GM-CSF (50 ng/ml), LPS (100 ng/ml), LTB<sub>4</sub> (1  $\mu$ M) and TNF- $\alpha$  (10 ng/ml) were added for 30 min at 37 °C. Samples were stained with the control antibodies APC/PE, and anti-CD62L on ice for 30 min. Change in expression of CD62L was measured on the BD Calibur flow cytometer. Data represents the mean of 4 separate experiments, each performed in triplicate. \* = significant compared to its own control, \*\* = significant when compared to the equivalent condition in the control set of experiments.



**Figure 3.2.4.4 Effect of R-roscovitrine and gliotoxin on neutrophil activation assessed by expression of CD11b induced by GM-CSF, LPS, LTB<sub>4</sub> and TNF- $\alpha$**

Neutrophils ( $5 \times 10^6$  /ml) in PBS + cations were pre treated for 30 min on a 37 °C shaking heat block as follows: (A) in PBS + cations alone (control), (B) in 20  $\mu$ M R-roscovitrine, (C) in 0.1  $\mu$ g/ml gliotoxin. To (A), (B) and (C) either PBS + (control), GM-CSF (50 ng/ml), LPS (100 ng/ml), LTB<sub>4</sub> (1  $\mu$ M) and TNF- $\alpha$  (10 ng/ml) were added for 30 min at 37 °C. Samples were stained with the control antibodies APC/PE, and anti-CD11b on ice for 30 min. Change in expression of CD11b was measured on the BD Calibur flow cytometer. Data represents the mean of 4 separate experiments, each performed in triplicate. \* = significant compared to its own control, \*\* = significant when compared to the equivalent condition in the control set of experiments.

Neutrophils ( $5 \times 10^6/\text{ml}$ , 250  $\mu\text{l}$  aliquots) in PBS (with  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) were pre-treated for 30 min with either R-roscovitine (20  $\mu\text{M}$ ), the NF- $\kappa\text{B}$  inhibitor gliotoxin (0.1  $\mu\text{g}/\text{ml}$ ), or PBS + cations (control), following which either PBS with  $\text{Ca}^{2+}/\text{Mg}^{2+}$  (control), GM-CSF (50  $\text{ng}/\text{ml}$ ), LPS (100 $\text{ng}/\text{ml}$ ),  $\text{LTB}_4$  (1  $\mu\text{M}$ ) and TNF- $\alpha$  (10  $\text{ng}/\text{ml}$ ) were added for 30 min to each of the three conditions, and the effects on the activation state of neutrophils measured on a BD Calibur flow cytometer. The concentrations of the reagents were optimised in previous experiments (data not shown) and because they represent the likely concentrations of the inflammatory mediators encountered by the neutrophil in the inflammatory milieu. Neutrophil activation was investigated as it is an important process in the functional progression of the cell. Upon encountering inflammatory stimuli neutrophils become 'primed', a term which encompasses an increase in neutrophil responsiveness, enabling chemotactic migration and upregulation of anti microbial capacity.

When a neutrophil becomes activated it stimulates polarisation of the cell, the cytoskeleton becomes reorganised causing the cell to spread and increase in size. This can be detected by an increase in forward scatter. As can be seen in Figure 3.2.4.2 (B) R-roscovitine when used alone or in conjunction with the other inflammatory agents does not cause any significant perturbation from the expected shape change response demonstrated in the control (Figure 3.2.4.2 (A)). However, gliotoxin (Figure 3.2.4.2 (C)) causes several alterations in activation as assessed by shape change when compared to the control; it significantly inhibits the ability of  $\text{LTB}_4$  and TNF- $\alpha$  to elicit the neutrophil shape change and partially reduces the extent of the shape change induced by GM-CSF (from an average of 70 % to an average of 47 %) indicating that gliotoxin impairs the ability of  $\text{LTB}_4$ , TNF- $\alpha$  and to a certain extent GM-CSF to prime the neutrophils into an active state. Independently neither R-roscovitine nor gliotoxin appear to be eliciting shape change when compared to the control.

Other markers of neutrophil activation investigated were CD62L and CD11b. The adhesion molecule CD62L (L-selectin), resides on the cell membrane of un-primed neutrophils playing a vital role in the first stages of neutrophil attachment to the endothelium, is shed upon neutrophil activation by proteolytic cleavage to make way for tight adhesion and

diapedesis, regulated by the  $\beta 2$  integrins such as CD11b. Upon activation CD11b is mobilised from the secondary granules within the neutrophil to the cell membrane also indicating activation of the cell.

Figure 3.2.4.3 shows the effects of R-roscovitine (B) and gliotoxin (C) on the cell activation marker CD62L. R-roscovitine has no discernable affect on CD62L expression either when alone or in conjunction with one of the four inflammatory mediators used, a slight reduction in CD62L expression of 21 % can be observed in the R-roscovitine/Control compared to Control/Control. However, when gliotoxin is examined it appears to have the ability to induce CD62L shedding, indicating activation of the neutrophil, causing a 53 % reduction in CD62L shedding compared to control condition. In addition gliotoxin also increases CD62L shedding when incubated in conjunction with LPS (a decrease of 29.1 % compared to the control). However, it inhibits the ability of TNF- $\alpha$  to stimulate CD62L shedding in neutrophils, evident by CD62L expression being 590 % higher in the gliotoxin TNF- $\alpha$  condition compared to its control. Gliotoxin also inhibits shedding of CD62L induced by GM-CSF (by 20.6 % compared to the control) and LTB<sub>4</sub> (by 24.9 % compared to the control).

CD11b expression does not appear to be altered as greatly by gliotoxin (Figure 3.4.4 (C)) as CD62L. However, when incubated in conjunction with TNF- $\alpha$  a significant reduction in CD11b expression (70.2 %) compared to the control can be observed. This indicates a notable inhibition of the ability of TNF- $\alpha$  to stimulate movement of the specific granules to the neutrophil cell surface membrane. A slight increase in expression of CD11b was evident in the gliotoxin conditions which were treated in conjunction with GM-CSF, LTB<sub>4</sub> and LPS of 25.7 %, 15.1 % and 12 % respectively when compared to the relevant controls. Again R-roscovitine (Figure 3.2.4.4 (B)) appears to be well tolerated by neutrophils in relation to CD11b regulation with results mirroring closely those of the control with only a minor deviation being apparent, a reduction of 29.2 % in the up-regulation of CD11b caused by TNF- $\alpha$  when compared to its control. However, this is still notably less significant an impact on CD11b levels than that of gliotoxin. The pro-survival agents LPS and LTB<sub>4</sub> in the control condition did not induce as great a level of CD11b upregulation as

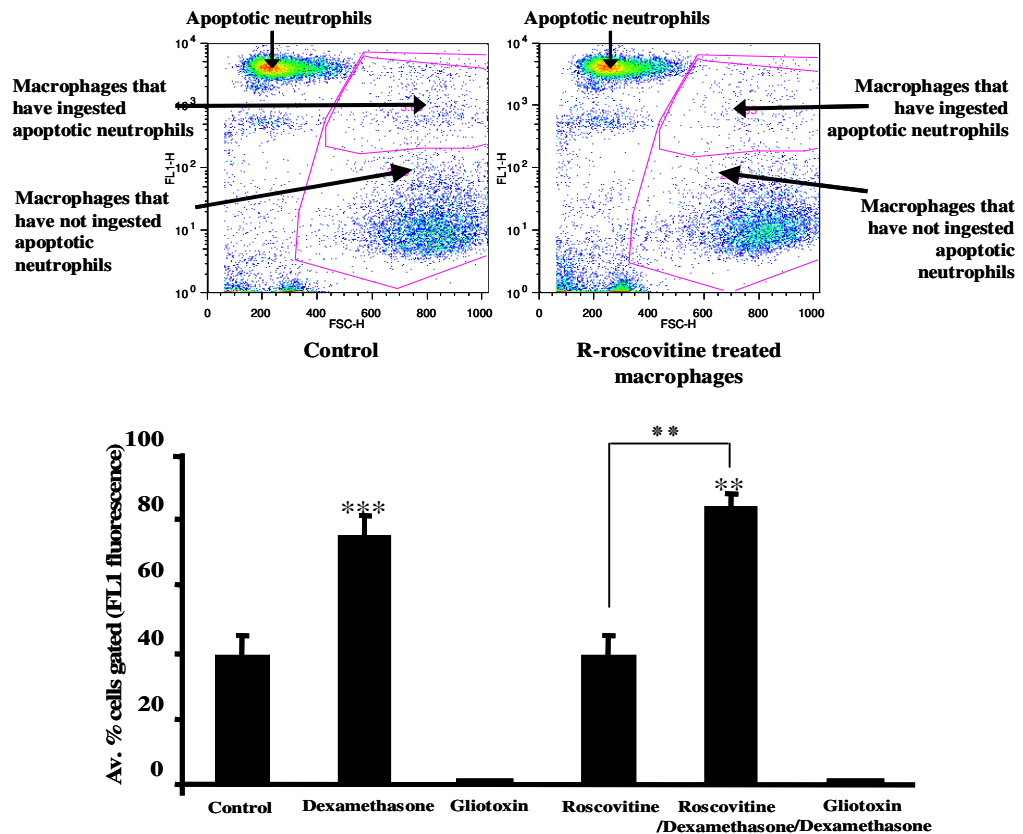
anticipated, this could be due to the concentrations at which the reagents were used or the duration of the incubation period was not optimum for the assay conducted.

These results are promising for possible pharmacological use of R-roscovitine as unlike other agents, such as gliotoxin, which induce apoptosis in neutrophils it appears that R-roscovitine is well tolerated by neutrophils in relation to pertinent markers of activation both alone or when in the presence of neutrophil survival factors often encountered at the inflammatory milieu, allowing their vital roles to be carried out unperturbed.

### **3.2.5 Effect of R-roscovitine on macrophage phagocytosis of apoptotic neutrophils *in vitro***

An important consideration to take into account when researching novel anti-inflammatory treatments for neutrophil dominant disorders, such as Cystic Fibrosis, through induction of neutrophil apoptosis is to ensure that the agents do not have a detrimental effect on the professional phagocytes. For example, if R-roscovitine were to induce macrophage apoptosis, an abundance of apoptotic neutrophils would accumulate at the site of inflammation, potentially entering secondary necrosis if they failed to be removed in a timely manner. This would result in the release of the neutrophils cytotoxic contents into the inflammatory milieu damaging tissues and exacerbating inflammatory disorders.





**Figure 3.2.5 Effect of R-roscovitine and gliotoxin on macrophage phagocytosis of apoptotic neutrophils in the presence and absence of dexamethasone**

Freshly isolated monocytes ( $4 \times 10^6$ /ml, 500  $\mu$ l) were cultured into macrophages over 5-7 days via a process of selective adherence in IMDM + penicillin/streptomycin supplemented with 10% autologous serum either in the absence (control) or presence of R-roscovitine 20  $\mu$ M; Dexamethasone 1  $\mu$ M; gliotoxin 0.1  $\mu$ g/ml; R-roscovitine 20  $\mu$ M + Dexamethasone 1  $\mu$ M; gliotoxin 0.1  $\mu$ g/ml + Dexamethasone 1  $\mu$ M. Neutrophils were aged by resuspending cells at  $20 \times 10^6$ /ml in IMDM + penicillin/streptomycin supplemented with 10 % autologous serum and incubating with CM Green at 37 °C, 5 % CO<sub>2</sub> for 20 h. The greened neutrophils were resuspended at  $4 \times 10^6$ /ml in IMDM and incubated with the washed macrophages for 1 h, 37 °C, 5 % CO<sub>2</sub>. Macrophages were then detached by incubation with 0.25 % trypsin / 1 mM EDTA for 15 min, 37 °C, cells were then incubated at 4 °C for 15 min and analysed by flow cytometry. Data represents mean of 5-7 separate experiments, each performed in triplicate.  $P < 0.01$  = \*\*,  $P < 0.001$  = \*\*\*.

Freshly isolated monocytes ( $4 \times 10^6$  /ml, 500  $\mu$ l) were plated and matured to macrophages over 5-7 days via a process of selective adherence in IMDM + penicillin/streptomycin supplemented with 10% autologous serum either in the absence (control) or presence of R-roscovitine 20  $\mu$ M; Dexamethasone 1  $\mu$ M; gliotoxin 0.1  $\mu$ g/ml; R-roscovitine 20  $\mu$ M + Dexamethasone 1  $\mu$ M; gliotoxin 0.1  $\mu$ g/ml + Dexamethasone 1  $\mu$ M. Neutrophils were aged by resuspending cells at  $20 \times 10^6$  /ml in IMDM + penicillin/streptomycin supplemented with 10 % autologous serum and incubated with CM Green to label the apoptotic cells, at 37 °C, 5 % CO<sub>2</sub> for 20 h. The effect of R-roscovitine on macrophages (exposed to R-roscovitine as monocytes and present during their maturation) was investigated to see if it impacts upon their ability to phagocytose apoptotic neutrophils. Dexamethasone was used as it is one of the most prevalent treatments for inflammatory disorders. It increases macrophage phagocytosis of apoptotic leucocytes (as shown in Figure 3.2.5), and also causes apoptosis in eosinophils making steroids highly effective treatments for allergic inflammatory diseases such as asthma. However, in neutrophil dominant disorders such as rheumatoid arthritis steroids, such as dexamethasone, whilst increasing macrophage phagocytosis of apoptotic cells aiding resolution actually prolongs neutrophil survival (as can be seen in Figure 3.2.3) allowing activated neutrophils to keep on secreting their cytotoxic arsenal and potentially exacerbating the inflammatory disorder. This makes the search for neutrophil apoptosis inducing agents for potential use in conjunction with glucocorticoids an important area of research. If this were successful a double pronged approach to the treatment of chronic neutrophil inflammation could be employed increasing neutrophil apoptosis and macrophage clearance of apoptotic cells.

As can be seen in Figure 3.2.5 gliotoxin had no macrophages present, the likely cause of which is death and premature detachment of the macrophages during the maturation process resulting in removal during the washing procedure. However, R-roscovitine when administered alone or in conjunction with dexamethasone did not affect the macrophages ability to phagocytose apoptotic neutrophils, even producing a slight increase of 9 % in the amount of cells phagocytosed when used in conjunction with dexamethasone, probably because of the increased presence of apoptotic neutrophils as a result of R-roscovitine.

Taken in conjunction, the results demonstrated in Figures 3.2.4 and 3.2.5 are in keeping with those of a successful novel treatment for neutrophil inflammatory disorders.

### 3.3 Discussion

The CDK inhibitor R-roscovitine is currently in phase II clinical trials for use as a novel chemotherapeutic agent for various cancers. CDK inhibitors are proving successful in the fight against cancer as cyclin dependent kinases are not only involved in regulation of the cell cycle but also play a crucial regulatory function in RNA polymerase II-mediated transcription (Krystof and Uldrijan, 2010).

However, cancer research is not the only field in which CDKis are eliciting prospective clinical benefits. Our laboratory has demonstrated that R-roscovitine also has potential therapeutic use as a novel treatment for inflammatory disorders. It can effectively induce neutrophil apoptosis *in vitro* in as little as 4 h despite the fact that neutrophils are terminally differentiated cells in which CDKs were thought to be redundant. The exact role which CDKs play in neutrophils and the mechanism by which R-roscovitine can induce apoptosis has yet to be elucidated; however, in this chapter it has been demonstrated that R-roscovitine can induce neutrophil apoptosis in as little as 4 h (as assessed by morphology and Annexin V/PI staining) and our laboratory has also demonstrated that R-roscovitine can induce neutrophil apoptosis in a time-and concentration-dependent manner (Rossi et al., 2006). The affects of R-roscovitine were compared to the effects of the apoptosis inducing fungal metabolite gliotoxin, which is known to induce apoptosis through inhibition of the NF- $\kappa$ B pathway and also 20S proteasome activity (Paugam et al., 2002). Previous research in our laboratory has demonstrated that the NF- $\kappa$ B pathway plays an important role in the inflammatory process and neutrophil apoptosis (Ward et al., 1999a) as it is hypothesised that the NF- $\kappa$ B pathway is involved in the prolongation of the neutrophil lifespan induced by the inflammatory mediator TNF- $\alpha$  and the bacterial toxin LPS. This was supported by the observation that by blocking protein synthesis the pro-apoptotic ability of TNF- $\alpha$  (at time points 8 h or less) is enhanced. This led to the hypothesis that TNF- $\alpha$  is triggering the NF- $\kappa$ B pathway and activating transcription of anti-apoptotic protein(s).

Furthermore, it has been demonstrated that NF- $\kappa$ B can stimulate transcription of pro-survival proteins such as BCX<sub>1</sub>, CIAP<sub>1</sub>, CIAP<sub>2</sub>, X-IAP and A1 therefore it is possible that

one or several of these proteins may regulate TNF- $\alpha$  induced neutrophil survival (Ward et al., 2004, Gyrd-Hansen and Meier, 2010). As the mechanism for gliotoxin induced apoptosis in neutrophils has been identified, gliotoxin was compared to R-roscovitine to see if there were any similarities between their effects on neutrophil apoptosis which could potentially implicate the NF- $\kappa$ B pathway in the mechanism of R-roscovitine induced apoptosis in neutrophils.

R-roscovitine was dissolved in the solvent DMSO. The DMSO present in the diluted experimental concentration (20 $\mu$ M) is 0.1%. At this concentration DMSO has been repeatedly shown within our laboratory to not have any effect on the parameters of neutrophil apoptosis investigated in this thesis, therefore the decision was taken not to control for DMSO in these experiments.

R-roscovitine was compared to an extensive list of survival factors and the neutrophil apoptosis inducing agent gliotoxin at 20 h, it was evident that the ability of R-roscovitine to induce neutrophil apoptosis at this time point was even greater than that of gliotoxin. Importantly our group has also demonstrated that R-roscovitine can overcome the anti-apoptotic effects of a plethora of inflammatory mediators, a promising finding in the hunt for a novel anti inflammatory agent (Rossi et al., 2006, Leitch et al., 2010).

When researching potential novel anti inflammatory agents it is essential that their general toxicity is examined, gliotoxin has been shown *in vivo* to possess a very narrow line between toxicity and therapeutic benefits (Fitzpatrick et al., 2000). Therefore, the general tolerability of R-roscovitine in comparison with gliotoxin was examined. An interesting observation that can be concluded from these studies is that R-roscovitine appears not affect the activation state of neutrophils, and importantly also does not affect the activation state of the neutrophil when co-administered with the key pro-survival agents GM-CSF, LPS, LTB<sub>4</sub> and TNF- $\alpha$ . This is an important finding as it infers that the recruitment and migration of neutrophils from the circulation to the site of inflammation will not be perturbed by R-roscovitine, as CD62L and CD11b play a pivotal role in the regulating neutrophil adhesion to the vascular endothelium. Also, it is imperative that neutrophils are

not untimely primed which renders them susceptible to activation. This has the potential to result in inappropriate release of the neutrophils cytotoxic arsenal and generation of superoxide anion, negatively impacting on the resolution of inflammation. If this process were augmented, as indicated by the result obtained from the gliotoxin treated conditions, it would imply disruption of the process of neutrophil recruitment to the sites of inflammation and infection. The lack of effect of R-roscovitine on pertinent markers of neutrophil activation is also supported by additional work conducted in our laboratory. It has been demonstrated that R-roscovitine does not affect the aggregation of neutrophils stimulated by either the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) or the phorbol ester PMA. In addition unlike the protein kinase C inhibitor Ro-31,8220 (Davis et al., 1989) R-roscovitine does not appear to affect the production of superoxide anion by PMA (as assessed by intracellular oxidation of dihydrorhodamine by flow cytometry). This assay also supports the data presented in this thesis as it confirmed that neutrophil shape change (assessed by forward scatter) was unaltered by R-roscovitine (Leitch et al., 2010).

Previous research by Rosales *et al.*, 2004 demonstrated that guanosine triphosphate (GTP)-dependent secretion in neutrophils is regulated by CDK5 (a small CDK which it has been suggested does not function in regulation of the cell cycle). Incubation with R-roscovitine prevented lactoferrin secretion (which resides in the specific (secondary) granules) and decreased levels of CD63 and CD66b localised to the surface membrane. From this it can be inferred that CDK5 in conjunction with its activator p35 (also present in neutrophils) is necessary for optimum GTP-regulated secretion from the azurophil (primary) and specific granules. As CD11b also resides in the specific granules it would therefore be expected that levels of CD11b would be reduced by incubation with R-roscovitine. However, as the results in Figure 3.2.4.3 show this was not the case and no significant reduction in CD11b was observed when compared to the controls. An explanation for this is that CDK5-p35 regulated GTP-induced secretion of azurophil and specific granules in neutrophils is being compensated by other mechanisms. This is supported by only partial inhibition being achieved in the Rosales study (Rosales et al., 2004) implying that GTP-induced secretion is redundant. Taken together these findings suggest that R-roscovitine possesses potential for

use as a novel anti-inflammatory agent in the treatment of neutrophil dominant inflammatory diseases.

Another interesting observation obtained from the results in this chapter is that the R-roscovitine does not appear to perturb monocyte derived macrophages ability to recognise and phagocytose apoptotic neutrophils, and in addition does not interfere with the ability of dexamethasone to up-regulate macrophage phagocytosis of apoptotic neutrophils (Liu et al., 1999) which is one of the main forms of treatment for inflammatory diseases. Furthermore dexamethasone has been previously been shown to prolong neutrophil life-span (also evident in Figure 3.3) (Meagher et al., 1996), which is not ideal in the treatment of neutrophil dominant inflammatory disorders such as Cystic Fibrosis. This is because inhibition of neutrophil apoptosis prolongs the presence of activated neutrophils at the site of inflammation increasing the likelihood of the release of their cytotoxic products and generation of superoxide anions. Prolonged neutrophil activation at sites of inflammation contributes to tissue damage and exacerbation of the inflammatory condition. Therefore, the ability of R-roscovitine to induce apoptosis in neutrophils but not affect the ability of the macrophage to phagocytose neutrophils that have undergone apoptosis, or the ability of glucocorticoids to up-regulate phagocytosis of the apoptotic cells is a promising finding.

The results obtained in this chapter contribute towards the search for novel treatments specifically targeted to neutrophil dominant inflammatory disorders. They indicate the potential for a possible double pronged approach through use of a neutrophil apoptosis inducing agents such as R-roscovitine in conjunction with glucocorticoids to aid resolution at the inflammatory loci and facilitate clearance of the apoptotic cells. Interestingly support for the use of R-roscovitine to aid resolution of inflammation has been proposed by Du *et al.* in 2009 when they concluded that R-roscovitine may help attenuate inflammation by inhibiting macrophage ability to produce LPS-induced nitric oxide helping quell some of the tissue damage that can occur in exacerbated inflammatory responses when regulatory mechanisms have gone awry (Du et al., 2009). Consolidation of the above provides hope that R-roscovitine could have pleiotropic effects benefiting the resolution of inflammation.

However, gliotoxin had a detrimental effect on the ability of macrophages to phagocytose apoptotic neutrophils culminating in a lack of data being obtained for the gliotoxin or the gliotoxin/ dexamethasone condition. There are several possible reasons for this, one is that gliotoxin is causing death of the macrophages either by apoptosis or necrosis. Waring elucidated that at high concentrations gliotoxin induces apoptosis of peritoneal macrophages mediated by reactive oxygen species (Waring et al., 1988). Further support for the fatal effect of gliotoxin on macrophages was reported by Anselmi K., *et al.*, 2007 who noted that gliotoxin induces death of hepatic macrophage Kupffer cells by apoptosis, followed by secondary necrosis (Anselmi et al., 2007). Alternatively, gliotoxin could be preventing adhesion of the macrophages to the plastic wells resulting cells becoming detached and lost during the washes. This is supported by findings from Müllbacher A., *et al.*, who demonstrated that aspergillus fumigatus produced metabolites, namely gliotoxin which prevented adhesion of thioglycollate-induced peritoneal macrophages to plastic and also suppressed their phagocytic ability (Mullbacher and Eichner, 1984). This could also account for our observation and is supported by Comera C. *et al.*, who also found that when gliotoxin was incubated with neutrophils it prevented their ability to phagocytose zymosan, caused reorganisation of the actin cytoskeleton resulting in cell shrinkage and removed filopodia (Comera et al., 2007). Therefore, gliotoxin may stimulate similar changes to occur in macrophages preventing their ability to phagocytose. Any one of the aforementioned or a combination of the above would explain the lack of data obtained in the conditions where macrophages were exposed to gliotoxin.

Interestingly Comera C. *et al.*, also observed that gliotoxin did not affect the ability of neutrophils to exocytose specific granules (one of the granules in which CD11b resides) (Comera et al., 2007). This supports the findings in Figure 3.2.4.3 in which the gliotoxin control condition caused little variance (a decrease of only 2.3 %) in the release of CD11b compared to the control condition. However, whilst on its own gliotoxin may not affect exocytosis of specific granules, when incubated in conjunction with survival factors, such as TNF- $\alpha$ , their ability to activate neutrophils (assessed by regulation of CD11b, CD62L and shape change) is drastically augmented, observed by deviations from the expected parameters shown in the control conditions. The implication of this is that on some level



gliotoxin is reducing the ability of the survival factors to induce exocytosis of at least one of the neutrophils stores of CD11b, be it the specific granules, tertiary granules, the secretory vesicles or a combination of all three. This is a serious implication when considering gliotoxin as a possible anti-apoptotic agent for therapeutic use.

To summarise, R-roscovitine is a powerful effector of neutrophil longevity inducing apoptosis in as little as 4 hours and dramatically increasing the expected rate of neutrophil apoptosis at 20 h compared to the untreated control. At 20 h R-roscovitine induces neutrophil apoptosis to a similar extent as the fungal metabolite gliotoxin. Unlike gliotoxin, R-roscovitine does not perturb the neutrophils activation response to key survival factors. Furthermore, in contrast to gliotoxin, R-roscovitine has no apparent detrimental effects on the ability of macrophages to phagocytose apoptotic neutrophils; and interestingly appears to enhance levels of phagocytosis. The likely cause of which is the increase in apoptotic neutrophils available for phagocytosis in the R-roscovitine treated conditions.

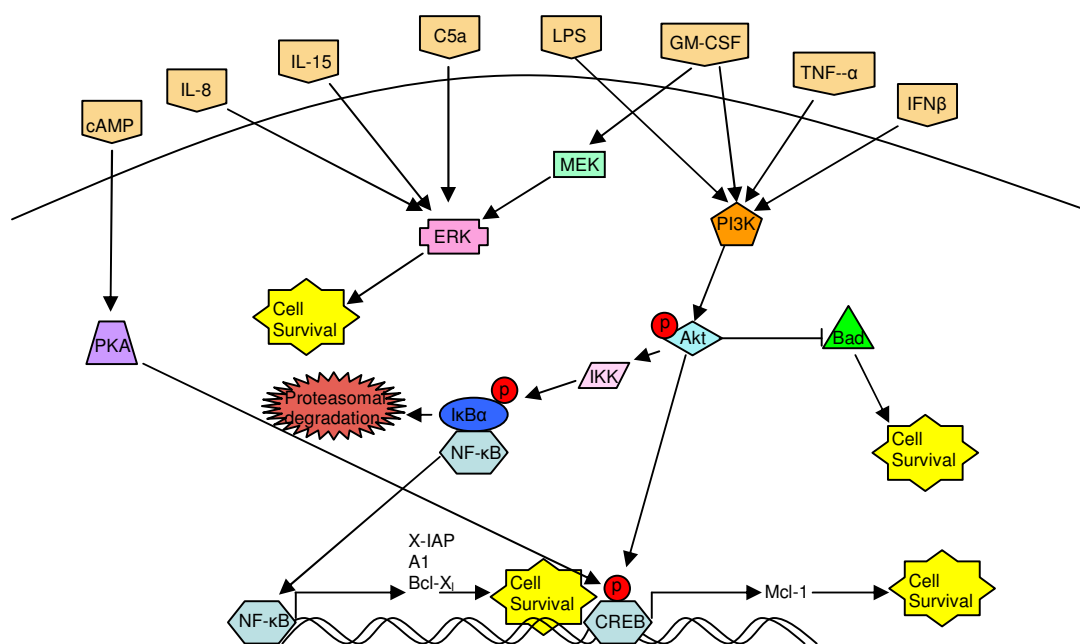
Whilst further studies are necessary to fully elucidate the full effects of R-roscovitine on neutrophil activation and on macrophage phagocytosis of apoptotic neutrophils the results here indicate greater exploration into the potential therapeutic use of R-roscovitine in neutrophil dominant inflammatory disorders is warranted.

## **Chapter 4**

### **The effect of R-roscovitine on NF- $\kappa$ B and NF- $\kappa$ B dependent and independent survival proteins in neutrophils.**

#### **4.1 Introduction**

The lifespan of the neutrophil can be regulated by inflammatory mediators using a plethora of often interlinking, sometimes redundant signaling pathways. Some of the most prevalent in the neutrophil are: phosphoinositide 3-kinase (PI3K) which can be triggered by LPS, GM-CSF, TNF- $\alpha$  and IFN $\beta$ ; the mitogen-activated protein kinases (MAPK) activated by IL-8, IL-15, C5a, GM-CSF and LPS; Protein Kinase A stimulated by cAMP; and NF- $\kappa$ B elicited by TNF- $\alpha$ , LPS and IL-1 (Riley et al., 2006, Leitch et al., 2009). It is the fluctuation in levels of these inflammatory stimuli which likely determine the longevity of the neutrophil through stimulation of the above signal transduction pathways. For example, inflammatory cytokines such as GM-CSF and bacterial toxins, including LPS, if elevated at sites of inflammation prolong the lifespan of the neutrophil. Whereas upon resolution, once invading bacteria have been removed and levels of inflammatory stimuli have decreased the balance is shifted towards neutrophil apoptosis. More details on the aforementioned pathways can be found in Chapter 1 (1.11 Intracellular signaling affecting granulocyte survival).



**Figure 4.1.1 Schematic representations of some of the signaling pathways involved in neutrophil survival and apoptosis**

*A depiction of some of the pro-inflammatory mediators and the pathways triggered within the neutrophil to promote survival. These pathways can share common points, for example GM-CSF activating PI3K and phosphorylating Akt can promote neutrophil survival through the inhibition of Bad, the activation of CREB to promote transcription of survival proteins such as Mcl-1, and the activation of NFκB pathway leading to transcription of anti-apoptotic proteins such as A1.*

#### **4.1.1 The NF-κB pathway**

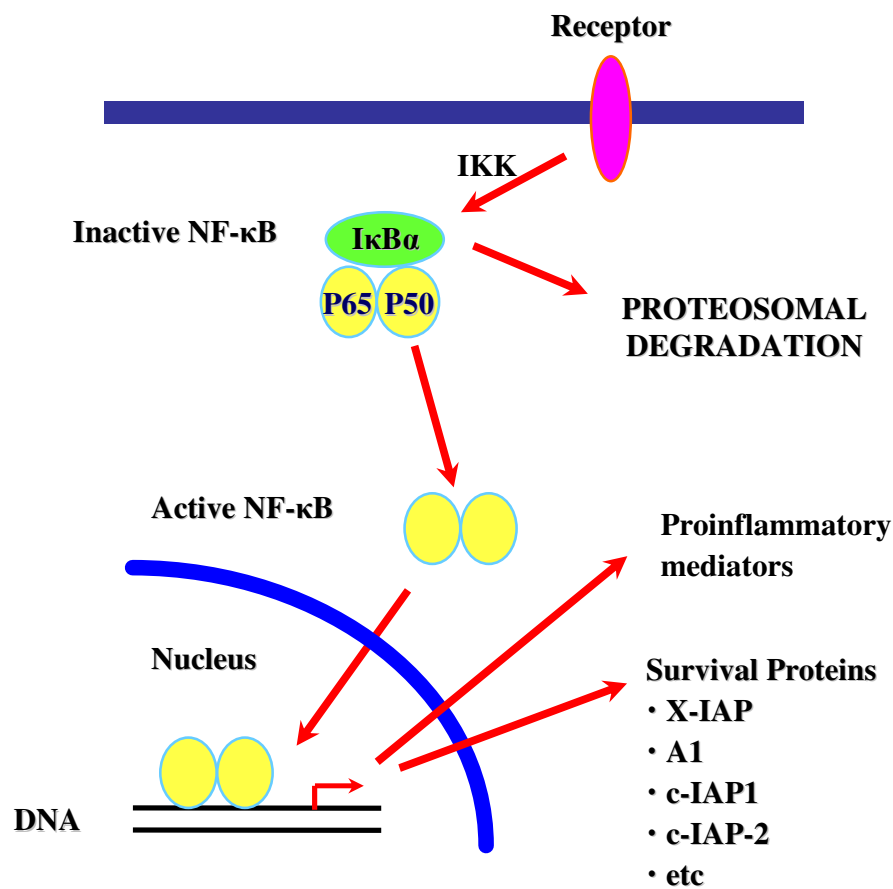
As mentioned above the NF-κB pathway is one of the key pathways implicated in regulating the fate of the neutrophil and is a pathway of particular interest in this thesis. The NF-κB dimer is a transcription factor comprised of either a homo or hetero-dimer of the following five subunits: p50/NF-κB1 (processed from the precursor p105); p52/NF-κB2 (derived from the precursor p100); p65/RelA; RelB; and c-Rel. It is negatively regulated by members of the IκB family (such as IκBα, IκBβ, p100 and p105) responsible for holding NF-κB in the cytoplasm by masking the nuclear localisation signal (Bakkar and Guttridge,

2010). Interestingly, however, the NF- $\kappa$ B pathway can provide negative feedback through stimulating transcription of the inhibitor I $\kappa$ B $\alpha$  (de Martin et al., 1993). In turn the I $\kappa$ B family is regulated by the I $\kappa$ B kinase complex (IKK), which is comprised of the kinases IKK $\alpha$ , IKK $\beta$  and the regulatory IKK $\gamma$ . IKK is responsible for controlling degradation of the inhibitory I $\kappa$ B protein, with IKK $\beta$  activating the canonical pathway (which also requires the presence of IKK $\gamma$ ) (Zandi et al., 1997) whereas, IKK $\alpha$  alone is necessary for the activation of the alternative pathway (Senftleben et al., 2001, Ghosh and Karin, 2002).

#### **4.1.1.1 Classical/Canonical NF- $\kappa$ B Pathway**

The classical pathway is the NF- $\kappa$ B pathway implicated in innate immunity and cell survival. It is also proposed to be the pathway activated in a variety of inflammatory diseases such as COPD and rheumatoid arthritis (Lawrence T. 2010). Furthermore the classical pathway has been associated with NF- $\kappa$ B dimers containing p65 or c-Rel (Karin and Ben-Neriah, 2000).

One of the most well documented stimuli for the classical NF- $\kappa$ B pathway is the cytokine TNF- $\alpha$ , which activates NF- $\kappa$ B through stimulation of the TNF receptors [a more detailed account of the TNF- $\alpha$  pathway can be found in Chapter 1 (1.9.2.1 Extrinsic Pathway)]. Transcription of target NF- $\kappa$ B genes are activated by phosphorylation of the I $\kappa$ B $\alpha$  by the IKK $\beta$  subunit of IKK. This marks I $\kappa$ B $\alpha$  for proteasomal degradation releasing the NF- $\kappa$ B hetero-dimer (as can be seen in Figure 4.1.2 and 4.1.3).



**Figure 4.1.2 Simplified pictorial representation of the classical NF-κB pathway (Ward et al., 1999a)**

*Activation of the classical NF-κB pathway, for example by stimulation of the TNFR1 and TNFR2 by TNF-α, and subsequent binding of TRADDs, can activate the transcription factor NF-κB by interaction with secondary adaptor molecules such as TNFR-associated factor-2 and RIP.*

#### **4.1.1.2 Alternative NF-κB Pathway**

For years it was considered that only one activation pathway existed for of NF-κB, the classical/canonical pathway. However, in 2000 Jimenez *et al.*, observed that NF-κB activation can occur in the absence of IκBα degradation and Senftleben *et al.*, 2001

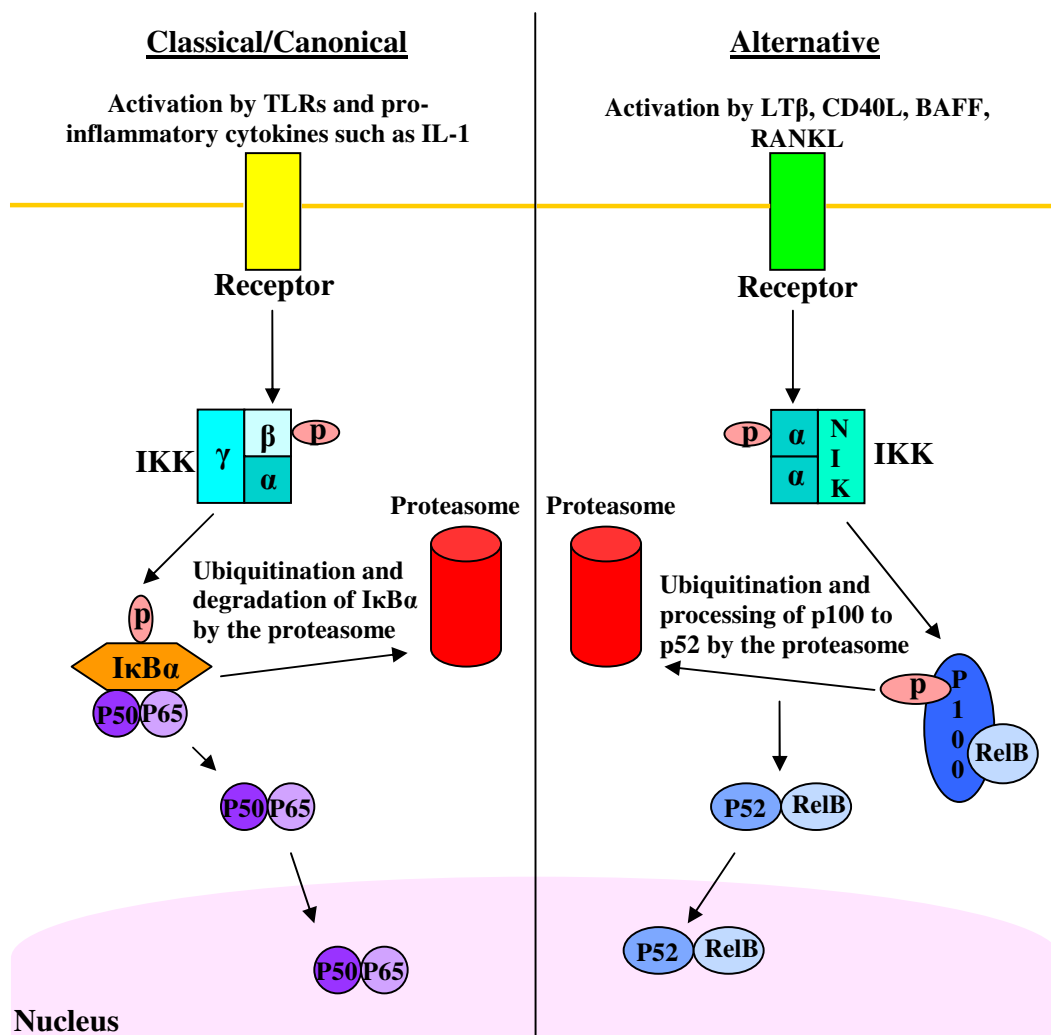
reported an alternative mechanism of NF- $\kappa$ B activation which involved the alternative IKK kinase IKK $\alpha$  (Figure 4.1.3) (Jiménez et al., 2000, Senftleben et al., 2001).

IKK $\alpha$  has an integral role in the alternative NF- $\kappa$ B activation pathway, which is independent of I $\kappa$ B $\alpha$  degradation and is regulated via processing of NF- $\kappa$ B p100 (alternatively known as NF- $\kappa$ B2) to form the NF- $\kappa$ B subunit p52. When in its native form p100 acts as an inhibitor of NF- $\kappa$ B, binding to cytoplasmic Rel-B. However, phosphorylation by IKK $\alpha$  induces interaction with NF- $\kappa$ B-inducing kinase (NIK) marking p100 for proteolytic processing to form p52 (Senftleben et al., 2001).

The alternative NF- $\kappa$ B pathway is implicated in the B-lymphocyte function and lymphoid organogenesis (Senftleben *et al.*, 2001), being activated by TNF-family cytokines but not TNF- $\alpha$  itself (Lawrence, 2009).

#### **4.1.2 NF- $\kappa$ B dependent and NF- $\kappa$ B independent survival proteins**

Many of the previously mentioned pathways prolong neutrophil lifespan through stimulating production of anti-apoptotic proteins. Neutrophils have been demonstrated to contain varied pro-survival proteins, several of which are regulated by NF- $\kappa$ B (X-IAP, A1, cIAP1 and cIAP2) (Gyrd-Hansen and Meier, 2010, Akgul et al., 2001). X-IAP, a member of the Inhibitor of Apoptosis protein family has been particularly well documented in relation to the neutrophil and for this reason was selected as one of the proteins for further investigation. One of the aims of this chapter is to determine if R-roscovitine could be influencing neutrophil survival via the disruption of the NF- $\kappa$ B pathway and subsequent survival protein induction.



**Figure 4.1.3 Diagrammatic representations of the classical and alternative NF- $\kappa$ B pathways. Modified from (Lawrence, 2009).**

The classical/canonical pathway can be activated by inflammatory mediators such as LPS, stimulating TLRs resulting of phosphorylation of I $\kappa$ B $\alpha$  by IKK marking I $\kappa$ B $\alpha$  for proteasomal degradation and freeing the NF $\kappa$ B hetero-dimer allowing translocation to the nucleus. The canonical pathway is implicated in inflammatory diseases such as COPD. The alternative pathway can be triggered by binding of lymphotoxin- $\beta$ , sequestering TNF receptor associated factors and activating NIK which phosphorylates the IKK $\alpha$  homo-dimer. This triggers processing of P100 to P52 by proteolysis, enabling the P52-RelB hetero-dimer to translocate to the nucleus. The alternative pathway is implicated in B-lymphocyte function.

#### **4.1.2.1 X-IAP**

As mentioned in Chapter 1, X-IAP exerts its effects by inhibiting the caspases 3, 7 and 9 by direct competitive inhibition (Eckelman et al., 2006). Interestingly, however, this does not appear to be its sole function. Whilst X-IAP is one of the genes transcribed by NF- $\kappa$ B (Stehlik et al., 1998) it also appears to provide a means of homeostatic regulation of the NF- $\kappa$ B pathway linking environmental stimuli to the response of the cell. For example, X-IAP has been demonstrated to provide positive feedback to the NF- $\kappa$ B pathway. This is achieved either through induced activation of the classical NF- $\kappa$ B pathway by X-IAP/TAK1 mediated activation of IKK $\beta$  (Hofer-Warbinek et al., 2000), or via binding the NF- $\kappa$ B inhibitor COMMD1 which regulates the stability of NF- $\kappa$ B sub-units (Galban and Duckett, 2010).

A fascinating discovery in mice has also implicated an important role for X-IAP in immune regulation. Mice deficient in X-IAP had reduced survival rates when infected with *Listeria monocytogenes* as X-IAP was required to potentiate NF- $\kappa$ B activation and extend JNK phosphorylation in response to bacterial infection. Furthermore, the presence of X-IAP appears necessary to obtain optimum pro-inflammatory cytokine production (Bauler et al., 2008).

#### **4.1.2.2 Mcl-1**

A survival protein which in humans is unlikely to be induced by NF- $\kappa$ B activation was also studied. Mcl-1 was chosen as it is known to play a critical role in the regulation of neutrophil apoptosis.

Transcription of the Mcl-1 gene can be regulated by a varied array of transcription factors. Akgul *et al.* characterised the Mcl-1 promoter region identifying the following potential transcription factor binding sites in the 5' region of the murine Mcl-1 gene: STAT; Ets; CRE-BP; and NF- $\kappa$ B. However, there is significant variation between the murine 5' promoter region of Mcl-1 and the human 5' promoter region. Through generation of a



series of mutants Akgul and colleagues concluded that it is doubtful that the human gene contains a true NF- $\kappa$ B binding site (Akgul et al., 2000b). STAT3 appears to be of particular importance in the regulation of Mcl-1 transcription being triggered by IL-6, IL-3 and VEGF. Furthermore, areas of inflammation are generally hypoxic and hypoxic conditions have been demonstrated to increase Mcl-1 transcription through hypoxia-inducible factor 1 $\alpha$  (Thomas et al., 2010). For more information on Mcl-1, including post translational modifications and the role of Mcl-1 in the neutrophil please see Chapter 1 (1.10 Neutrophil Survival Proteins).

The aim of this chapter was to attempt to unravel the pathway(s) through which R-roscovitine signals to induce neutrophil apoptosis. It has been previously proposed by Dey et al in 2008 that R-roscovitine inhibits IKK activity in tumorigenic cells thereby suppressing activation of NF- $\kappa$ B (Dey et al., 2008). Therefore, we chose to study the effect of R-roscovitine on NF- $\kappa$ B activation to explore the possibility of a similar mode of action in the inflammatory neutrophil. This was extended by examining the effect of R-roscovitine on levels of the NF- $\kappa$ B regulated survival protein X-IAP. Furthermore, the effect of R-roscovitine on the survival protein Mcl-1 was also considered as it has also been reported that R-roscovitine interferes with RNA pol II disrupting transcription of the highly liable Mcl-1 protein (Fromaget and Cook, 2007).

## 4.2 Results

### 4.2.1 Effect of mediators of neutrophil survival and R-roscovitine on I $\kappa$ B $\alpha$ in neutrophils.

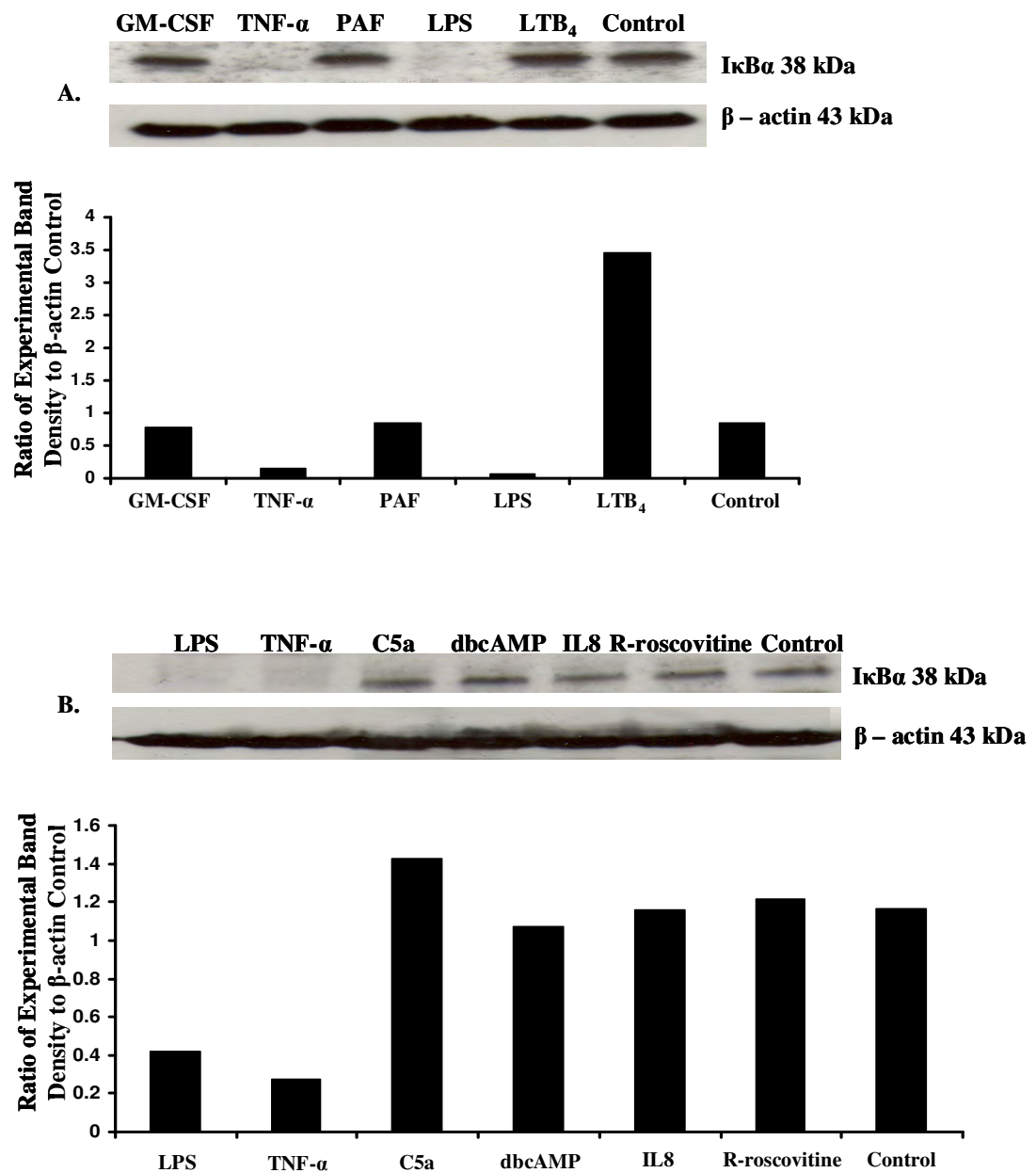
As it has been widely reported that NF- $\kappa$ B plays a key role in the initiation of neutrophil apoptosis, this pathway was chosen as a hopeful candidate in which to start investigation into the mechanism through which R-roscovitine induces apoptosis.

In order to establish/confirm agents that result in degradation of the I $\kappa$ B $\alpha$  inhibitor of NF- $\kappa$ B in neutrophils the following investigations were performed. This subsequently allowed us to determine if the chosen agents could be activating the canonical pathway in neutrophils.

Freshly isolated neutrophils at  $5 \times 10^6$  / ml in IMDM plus 10 % autologous serum were incubated with either 50 ng/ ml of GM-CSF, 10 ng/ml TNF- $\alpha$ , 1  $\mu$ M PAF, 100 ng/ml LPS, 1  $\mu$ M LTB $_4$  or IMDM plus 10% autologous serum (control) for 15 min on a shaking heat-block at 37 °C before lysing and performing SDS PAGE on a 12 % gel. Figure 4.2.1 A. shows that LPS and TNF- $\alpha$  both cause degradation of I $\kappa$ B $\alpha$ , demonstrated by an absence of a band in the appropriate lanes compared to the control  $\beta$ -actin blot (where bands are present demonstrating equal loading). Therefore, it can be concluded that they free the NF- $\kappa$ B heterodimers from inhibition by I $\kappa$ B $\alpha$ , leaving it able to translocate to the nucleus. The cytokine GM-CSF and the lipid mediators PAF and LTB $_4$  do not cause degradation of I $\kappa$ B $\alpha$ , observed by the presence of bands in the appropriate lanes and therefore it can be concluded that they do not cause activation of NF- $\kappa$ B via degradation of I $\kappa$ B $\alpha$ , at least under the conditions used in this experiment.

To discover if R-roscovitine causes I $\kappa$ B $\alpha$  degradation in neutrophils and therefore could be acting, at least in part, through NF- $\kappa$ B activation, the above experiment was repeated only this time the experimental conditions used were Control (IMDM plus 10% autologous serum), 100 ng/ml LPS, 10 ng TNF- $\alpha$ , 1  $\mu$ M C5a, 0.2mM dbcAMP, 100 nM IL8 and 20

$\mu$ M R-roscovitine. As can be seen in Figure 4.2.1 B. LPS and TNF- $\alpha$  both cause degradation of I $\kappa$ B $\alpha$  (as shown in A.), whilst complement component C5a, dbcAMP and the chemokine IL8 do not. Interestingly, R-roscovitine does not appear to activate degradation of I $\kappa$ B $\alpha$ , indicating that the NF- $\kappa$ B hetero-dimer remains under its inhibition in the cytoplasm of the neutrophil. This implies that apoptosis inducing effect of R-roscovitine in neutrophils is not through the direct activation of NF- $\kappa$ B by the canonical pathway.



**Figure 4.2.1 Effect of inflammatory mediators on IκBα degradation in neutrophils.**

**Figure 4.2.1 Effect of inflammatory mediators on I $\kappa$ B $\alpha$  degradation in neutrophils.**

*A. Neutrophils ( $5 \times 10^6$  /ml) resuspended in IMDM with 10% autologous serum were incubated for 15 min at 37 °C on a shaking heat block with either IMDM alone (control), GM-CSF (50 ng/ml), TNF- $\alpha$  (10 ng/ml), PAF (1  $\mu$ M), LPS (100 ng/ml) or LTB $_4$  (1  $\mu$ M). Cells were then lysed (as described in Chapter 2 Materials and Methods) and the lysates separated on a 12 % separating gel by SDS electrophoresis. Western Blots were performed onto PVDF membrane and blocked in 5 % Marvel / TBS / 0.1 % Tween-20 for 1 h at room temperature. Blots were washed 3 x 5 min. in TBS / 0.1 % Tween-20, then incubated with the Santa Cruz antibody I $\kappa$ B $\alpha$  (C21) SC-371 diluted 1:200 in 5 % Marvel / TBS / 0.1 % Tween-20 overnight at 4 °C with gentle agitation. Blots were washed and then incubated with the secondary antibody goat anti rabbit HRP-conjugated diluted 1:2000 in 5% Marvel/TBS/0.1% Tween-20 for 1 h. at room temperature. Blots were visualised using electrochemiluminescence. Data represents results from 3 separate experiments.*

*Histogram represents the ratio of the experimental I $\kappa$ B $\alpha$  bands to the relevant  $\beta$ -actin control of the blots depicted in Figure 4.2.1 A, analysed by Image J.*

*B. Same protocol as A. Concentration of additional reagents: Roscovitine (20 $\mu$ M), C5a (1 $\mu$ M), dbcAMP (0.2mM), IL8 (100nM). Data represents results from 3 separate experiments.*

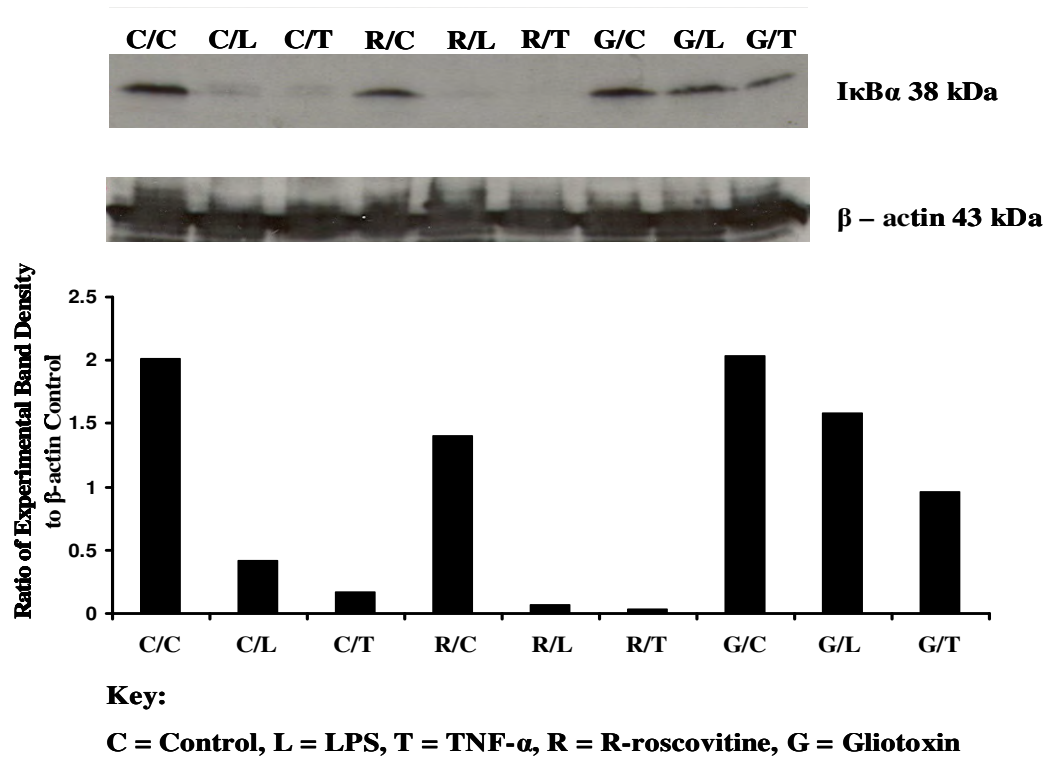
*Histogram represents the ratio of the experimental I $\kappa$ B $\alpha$  bands to the relevant  $\beta$ -actin control of the blots depicted in Figure 4.2.1 B, analysed by Image J.*

#### **4.2.2 Effect of R-roscovitine on the ability of LPS and TNF- $\alpha$ to activate NF- $\kappa$ B.**

Given the results obtained in the previous experiment, that R-roscovitine does not directly induce degradation of I $\kappa$ B $\alpha$ , it was decided to investigate if R-roscovitine could be inhibiting the ability of pro-survival agents, such as LPS and TNF- $\alpha$ , to pro-long neutrophil lifespan through activation of NF- $\kappa$ B and subsequent transcription of survival proteins such as X-IAP. Gliotoxin was used as a positive control as it has been previously demonstrated to inhibit LPS and TNF- $\alpha$  induced activation of NF- $\kappa$ B by preventing the loss of I $\kappa$ B $\alpha$  (Ward et al., 1999a).

Neutrophils at  $5 \times 10^6$  /ml in IMDM plus 10 % autologous serum were incubated for 30 min with either IMDM + 10 % autologous serum (control), R-roscovitine (20  $\mu$ M) or gliotoxin (0.1  $\mu$ g/ml). Following this either IMDM + 10 % autologous serum (control), LPS (100 ng/ml), or TNF- $\alpha$  (10 ng/ml) was added and samples were incubated for a further 15 min.

From figure 4.2.2 it can be seen that the control/control condition did not cause degradation of I $\kappa$ B $\alpha$ , whilst the control/LPS and the control/TNF- $\alpha$  conditions did. Furthermore, the gliotoxin/control condition did not lead to I $\kappa$ B $\alpha$  degradation, and when gliotoxin was pre-incubated with either LPS or TNF- $\alpha$  degradation of I $\kappa$ B $\alpha$  was prevented indicating that NF- $\kappa$ B was not activated. R-roscovitine, however, again did not lead to degradation of I $\kappa$ B $\alpha$  in the R-roscovitine/control condition and in addition it did not prevent the ability of LPS or TNF- $\alpha$  to degrade I $\kappa$ B $\alpha$ . This therefore indicates that R-roscovitine does not induce apoptosis in the same way as gliotoxin, via inhibition of I $\kappa$ B $\alpha$ .



**Figure 4.2.2 Effect of inflammatory mediators on IκBα degradation in neutrophils pre-treated with either R-roscovitine or gliotoxin.**

Neutrophils ( $5 \times 10^6$  /ml) resuspended in IMDM with 10 % autologous serum were incubated for 30 min at 37 °C on a shaking heat block with either IMDM alone (control conditions), R-roscovitine (20 μM), or gliotoxin (0.1 μg/ml). These conditions were then treated for 15 min at RT with either IMDM alone (control), LPS (100 ng/ml), or TNF-α (10 ng/ml). Cells were then lysed (as described in Chapter 2 Materials and Methods) and the lysates separated on a 12% separating gel by SDS electrophoresis. Western Blots were then performed onto PVDF membrane. These were blocked in 5 % Marvel / TBS / 0.1 % Tween-20 for 1 h at room temperature. The blots were washed 3 x 5 min in TBS / 0.1 % Tween-20, then incubated with the Santa Cruz antibody IκBα (C21) SC-371 diluted 1:200 in 5% Marvel / TBS / 0.1 % Tween-20 overnight at 4 °C with gentle agitation. Blots were washed and then incubated with the secondary antibody, goat anti-rabbit HRP-conjugated, diluted 1:2000 in 5% Marvel / TBS / 0.1 % Tween-20 for 1 h at room temperature. Blots were visualised using electrochemiluminescence. Data represents results from 4 separate experiments. Histogram represents the ratio of the experimental IκBα bands to the relevant β-actin control, analysed by Image J.

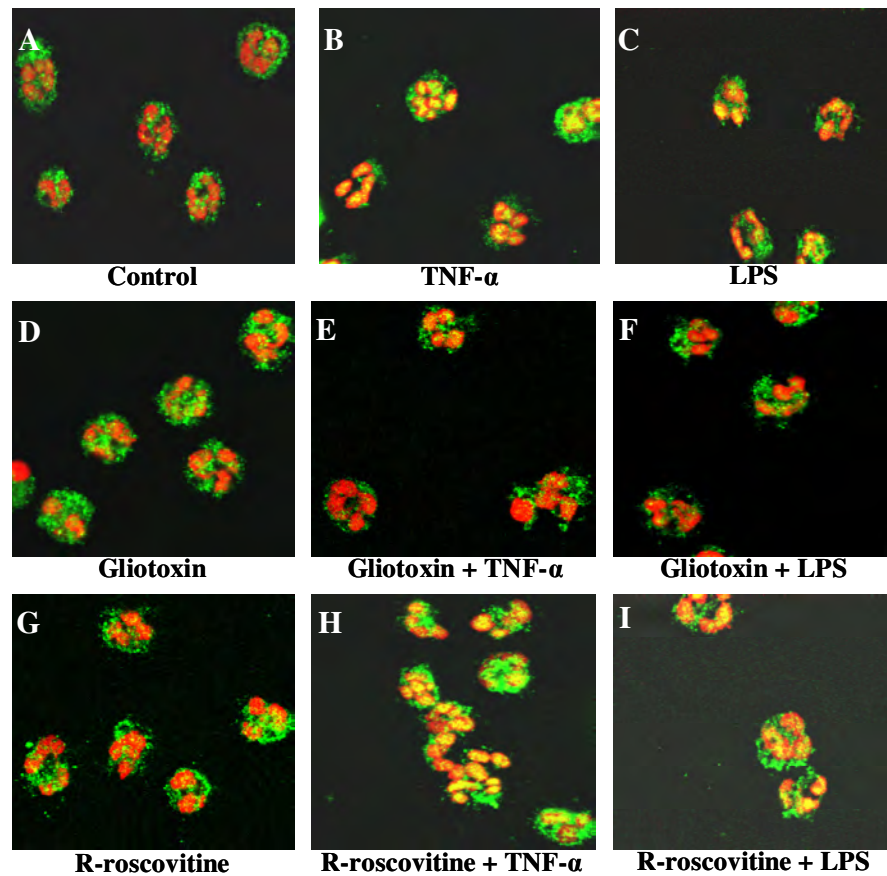
#### **4.2.3 Confocal micrographs of p65 translocation in neutrophils.**

As it has been previously reported that on rare occasions NF- $\kappa$ B activation can occur in the absence of I $\kappa$ B $\alpha$  degradation (Jiménez et al., 2000), the p65 subunit of the NF- $\kappa$ B heterodimer was stained using alexa-fluora green whilst the nucleus was stained red with PI, allowing the translocation of the p65 subunit upon NF- $\kappa$ B activation to be followed. This was performed to ensure that R-roscovitine was not triggering NF- $\kappa$ B activation independently I $\kappa$ B $\alpha$  degradation.

The same conditions were used as the previous experiment. Neutrophils ( $2.5 \times 10^6$  /ml) were cultured in IMDM with 10 % autologous serum either in the absence of any additional agents (control), R-roscovitine (20  $\mu$ M) or gliotoxin (0.1  $\mu$ g/ml) for 30 min. Samples were then incubated with either IMDM + 10 % autologous serum (control), LPS (100 ng/ml), or TNF- $\alpha$  (10 ng/ml) for a further 15 min before staining with propidium iodide and anti-human anti-p65 antibody (1:50).

In cells where the p65 subunit of NF- $\kappa$ B has not translocated the cytoplasm will remain green and the nucleus red. However, upon activation of NF- $\kappa$ B, translocation of p65 to the nucleus will result in the nucleus turning yellow.





**Figure 4.2.3 Effect of R-roscovitine and gliotoxin on LPS and TNF- $\alpha$  mediated movement of p65.**

***Confocal micrographs (Plan-Apochromat 100 x / 1.4 oil Ph 3)***

*Neutrophils ( $2.5 \times 10^6$  /ml) were cultured in IMDM + 10 % autologous serum with either media (Control), 0.1  $\mu$ g/ml of gliotoxin, or 20  $\mu$ M R-roscovitine for 45 min. Either IMDM with 10 % autologous serum (Control), 10 ng/ml TNF- $\alpha$  or 100 ng/ml LPS was added after 30 min for the remaining 15 min of the incubation. Samples were stained with propidium iodide and anti-human anti-p65 antibody (1:50). Data represents results from 3 separate experiments.*

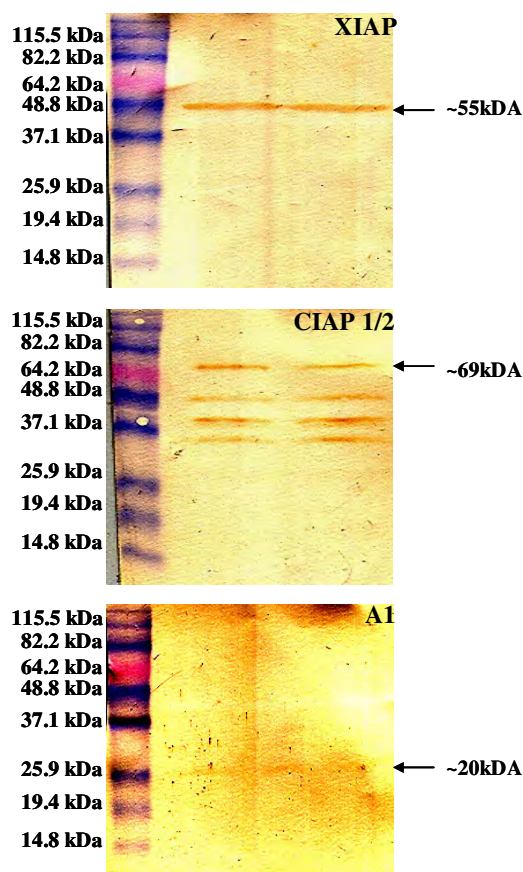
As can be seen in Figure 4.2.3 (A) The control demonstrates that there has been no NF- $\kappa$ B activation as the cytoplasm remains green indicating that the p65 subunit, and therefore NF- $\kappa$ B, is localised in the cytoplasm and has not translocated to the nucleus, which remained red. In panel (B) when neutrophils were treated with TNF- $\alpha$  the nuclei have distinct yellow patches indicating that the green p65 has translocated into the red nucleus. The same can be seen in panel (C) (LPS) therefore, again p65 has been released from its inhibitor and allowed to translocate to the nucleus. The positive control, gliotoxin, also yielded the same results as the previous experiment as demonstrated in panel (D) where neutrophils treated with gliotoxin exhibited a green cytoplasm and a red nucleus i.e. no translocation, this was also seen in panels (E) and (F) (gliotoxin/TNF- $\alpha$  and gliotoxin/LPS respectively). However, when the neutrophils were treated with R-roscovitine (G) the nuclei remain red and the cytoplasm green demonstrating that NF- $\kappa$ B is not being activated by R-roscovitine in the absence of I $\kappa$ B $\alpha$  degradation. Furthermore, these results confirmed that R-roscovitine was not preventing the translocation of p65 when incubated with either TNF- $\alpha$ , or LPS visible by the yellow patches of co-localisation within the nuclei of panels (H) and (I) respectively.

#### **4.2.4 Identification by Western Blotting of cIAP, X-IAP and A1 in human neutrophil lysates.**

Whilst all cells do not express all anti-apoptotic proteins it has been reported that neutrophils express cIAP, X-IAP and A1 (Riley et al., 2006). To confirm the presence of these inhibitors of apoptosis at a protein level in human neutrophils ( $5 \times 10^6$  /ml) in PBS (without  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$ ) were lysed immediately. Samples were then run on a 12 % gel and Western Blotting analysis was conducted according to the protocol in Chapter 2. Blots were blocked for 2 h in 5 % Marvel / TBS / 0.1 % Tween-20, and incubated with the primary antibody overnight. The antibodies used were at the following concentrations (A1 sc-8351 1:200, cIAP1/2 sc-12410 1:200, X-IAP AF8221 1:1000). Blots were visualised by diaminobenzidine staining (DAB).

As can be seen in figure 4.2.4 the presence of X-IAP, cIAP1/2 and A1 can be confirmed in human neutrophils. X-IAP can be observed on SDS-PAGE at a molecular weight of 55

kDa, cIAP1 at a molecular weight of 70 kDa and cIAP2 at 68 kDa (a combined antibody from Santa Cruz was used which visualises cIAP1 and 2 together in one band at approximately 69 kDa). The observation that A1 appears at a slightly higher molecular weight when run on SDS-PAGE than the anticipated 20 kDa was also evident. A possible explanation for this is that the protein may have undergone post-translational modifications.



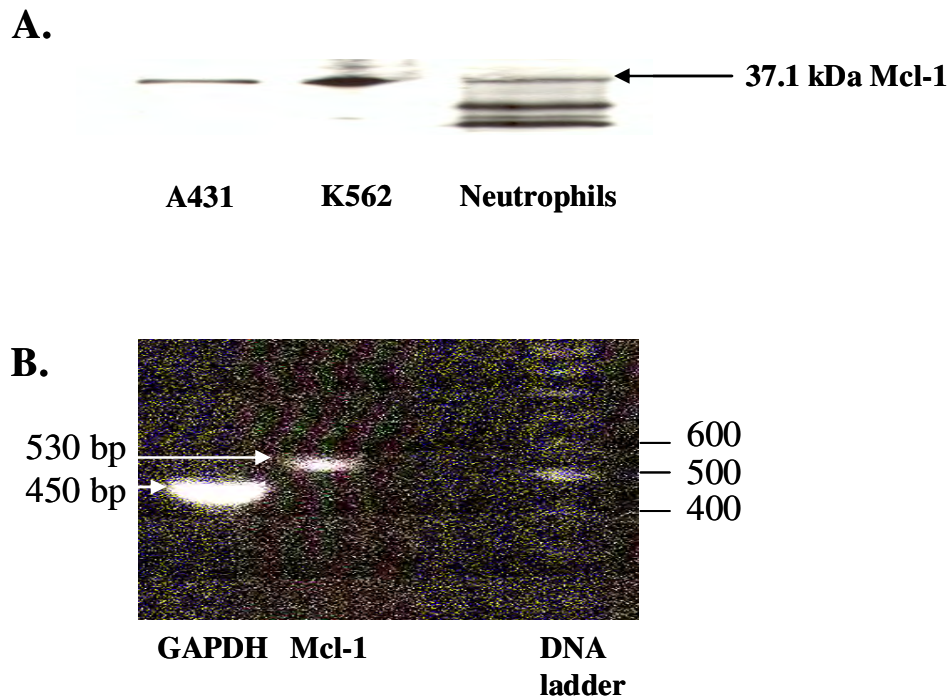
**Figure 4.2.4 Identification of the anti-apoptotic proteins cIAP, X-IAP and A1 in neutrophil lysates.**

*Freshly isolated human neutrophils ( $5 \times 10^6$  /ml) in PBS (without  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$ ) were lysed immediately. Samples were then run on a 12 % gel and Western Blotting analysis performed. Primary antibodies were incubated at the following concentrations overnight (A1 sc-8351 1:200, cIAP1/2 sc-12410 1:200, X-IAP AF8221 1:1000). Blots were visualised by DAB staining.*

#### **4.2.5 Identification of Mcl-1 mRNA and protein in human neutrophil lysate.**

A protein of particular interest in this thesis is Mcl-1. This is because the function of Mcl-1 has been particularly well documented, in the human neutrophil, as being pivotal to the initiation of apoptosis (Edwards et al., 2004). Therefore, it is highly likely that R-roscovitine will exert some of its apoptosis inducing ability through regulation of this protein, be it directly or indirectly. The presence of Mcl-1 in neutrophils has been observed at both the translational and transcriptional level (Figure 4.2.5). Western Blot analysis was used to identify the Mcl-1 protein. Freshly isolated human neutrophils were lysed immediately at a concentration of  $5 \times 10^6$  cells/ml in PBS (without  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ). Samples were run on a 12 % gel and western blotted according to the protocol in Chapter 2. The cell lines A431 (human skin carcinoma squamous cells) and K562 (chronic myelogenous leukemia cells) were run as positive controls. The blots were as transferred onto PVDF and incubated with the primary antibody (S19: SC-189) at 1:200 for 1 h. The band demonstrating Mcl-1 ran at approximately 40 kDa (the molecular weight of Mcl-1 is 37 kDa). However, in the neutrophil two further bands are evident at a slightly lower molecular weight, reasons for this will be discussed in the results section.

The presence of Mcl-1 at the transcriptional level was confirmed by the use of reverse transcription polymerase chain reaction (RT-PCR) as detailed in Chapter 2. RNA was harvested from freshly isolated neutrophils ( $25 \times 10^6$  /ml) and converted into cDNA. The PCR process was then carried out, the product of which was run on a 1 % agarose gel and visualised by ethidium bromide staining under UV light. The house keeping gene GAPDH, known to be expressed at significant levels in human neutrophils, was used as a positive control to demonstrate the effective nature of the RT-PCR process. The Mcl-1 band was present at 530 base pairs.



**Figure 4.2.5 Identification of Mcl-1 in neutrophils by Western Blot analysis and RT-PCR**

*A) Freshly isolated human neutrophils ( $5 \times 10^6$  /ml) in PBS (without  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$ ) were lysed immediately. The cell lines A431 and K562 were run as positive controls. Samples were run on a 12 % gel and analysed by Western Blot according to the protocol in Chapter 2. The blots were as transferred onto PVDF and blocked in 5 % Marvel / TBS / 0.1 % Tween-20 for 1 h at room temperature. Blots were incubated with the primary antibody (S19: SC-819) at 1:200 for 1 h. They were visualised using electrochemiluminescence. Data represents results from 3 separate experiments.*

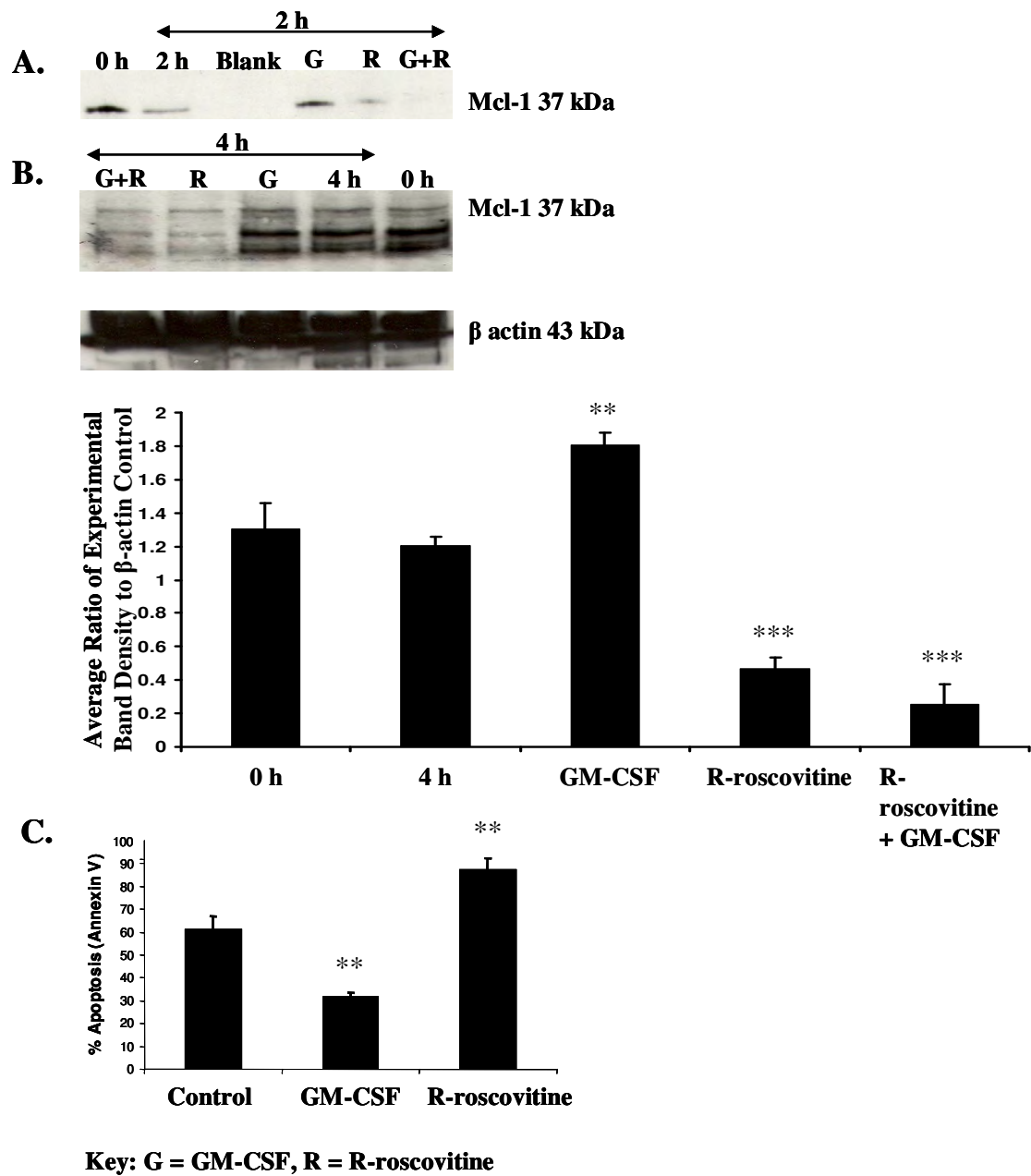
*B) RNA was harvested from freshly isolated neutrophils ( $25 \times 10^6$  / sample) and converted into cDNA. The PCR process was then carried out according to the protocol in Chapter 2. The PCR product was run on a 1 % agarose gel and visualised by ethidium bromide staining under UV light. The house keeping gene GAPDH was used as a positive control.*

#### **4.2.6 Effect of R-roscovitine on GM-CSF induced neutrophil survival and Mcl-1**

In order to investigate if the CDKi R-roscovitine could be influencing neutrophil survival by affecting levels of the important regulator of neutrophil apoptosis Mcl-1, expression of the Mcl-1 protein was studied using Western Blot analysis. As it had been previously demonstrated in neutrophils that Mcl-1 protein levels are maintained by the neutrophil survival mediator GM-CSF (Derouet et al., 2004) it was decided to investigate whether treatment of neutrophils with R-roscovitine had any effect on the ability of GM-CSF to maintain Mcl-1 levels. This is of particular importance as GM-CSF levels are increased at sites of inflammation, and is one of the many factors attributed to increased longevity of the neutrophil at inflammatory foci.

Freshly isolated human neutrophils at a final concentration of  $5 \times 10^6$  cells/ml in IMDM + 10% autologous serum were either lysed immediately (0h) or after 2 h incubation in a shaking water bath at 37 °C either with GM-CSF (50 ng/ml), R-roscovitine (20 µM), GM-CSF (50 ng/ml) + R-roscovitine (20 µM) or IMDM 10% autologous serum (2 h control). Samples were resolved on a 12 % gel and analysed by western blotting according to the protocol in Chapter 2 (as can be seen in Figure 4.2.6 A). The same experiment was also performed with a 4 h incubation (Figure 4.2.6 B.). Figure 4.2.6.C. depicts GM-CSF induced neutrophil survival at 20 h, as assessed by flow cytometric analysis of Annexin V binding.

Interestingly, it was observed that in as little as 2h, the level of Mcl-1 protein in neutrophils declined markedly; tipping the delicate balance of the Bcl-2 family member proteins towards apoptosis. Upon incubation with R-roscovitine Mcl-1 protein levels were also reduced and to a slightly larger extent than if R-roscovitine was not present. However, this decline in Mcl-1 protein was prevented by the addition of GM-CSF. Furthermore, when R-roscovitine was co-administered with GM-CSF, R-roscovitine inhibited the GM-CSF mediated upregulation of Mcl-1 protein levels. This is a significant finding when contemplating the use of R-roscovitine as a novel anti-inflammatory agent, as GM-CSF is often found elevated in diseases such as COPD and allergic rhinitis, exacerbating disease by conferring neutrophil survival.



**Figure 4.2.6** Effect of R-roscovitrine on GM-CSF induced neutrophil survival and the anti-apoptotic protein Mcl-1

**Figure 4.2.6 Effect of R-roscovitine on GM-CSF induced neutrophil survival and the anti-apoptotic protein Mcl-1**

*A) Mcl-1 levels in neutrophils (final concentration  $5 \times 10^6$  /ml) were measured by Western blot analysis immediately after isolation (0 h) or after 2 h incubation in a shaking water bath at 37 °C either with GM-CSF (50 ng/ml), R-roscovitine (20  $\mu$ M), GM-CSF (50 ng/ml) + R-roscovitine (20  $\mu$ M) or IMDM + 10 % autologous serum (2 h control). n = 4.*

*B) Mcl-1 levels in neutrophils (final concentration  $5 \times 10^6$  /ml) were measured by Western blot analysis using the same conditions as A) only with an incubation period of 4 h. Blots were stripped and reprobed for  $\beta$ -actin to check for equal loading. The histogram represents the average ratio of the experimental Mcl-1 bands to the relevant  $\beta$ -actin controls, analysed by Image J (n = 4)  $P < 0.01 = **$ ,  $P < 0.001 = ***$ .*

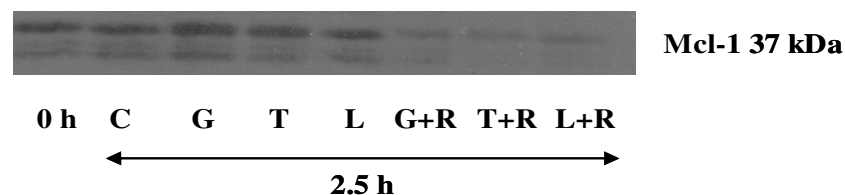
*C) Histogram showing percentage of apoptotic neutrophils as measured by Annexin V binding and flow cytometry. Neutrophils were plated at a final concentration of  $5 \times 10^6$  /ml in IMDM + 10 % autologous serum and incubated for 20 h at 37 °C, 5 % CO<sub>2</sub> in either IMDM + 10 % autologous serum (control), GM-CSF (50 ng/ml) or R-roscovitine (20  $\mu$ M). GM-CSF caused a significant decrease in apoptosis and R-roscovitine caused a significant increase in apoptosis ( $P < 0.01 = **$ ,  $P < 0.001 = ***$ ). n = 3 and 4 respectively.*



#### **4.2.7 Effect of R-roscovitine on GM-CSF, TNF- $\alpha$ and LPS induced neutrophil survival and the anti-apoptotic protein Mcl-1**

Given the above observation that R-roscovitine can prevent GM-CSF induced neutrophil survival by decreasing the protein level of Mcl-1 we chose to investigate next if this phenomenon was also evident in cells treated with other pro-inflammatory mediators, namely TNF- $\alpha$  and LPS. Freshly isolated human neutrophils at a final concentration of  $5 \times 10^6$  cells/ml in IMDM + 10% autologous serum were either lysed immediately (0 h), or lysed after 2.5 h following incubation in a shaking water bath at 37 °C either with GM-CSF (50 ng/ml), TNF- $\alpha$  (10 ng/ml), LPS (100 ng/ml), GM-CSF (50 ng/ml) + R-roscovitine (20  $\mu$ M), R-roscovitine (20  $\mu$ M) + TNF- $\alpha$  (10 ng/ml), R-roscovitine (20  $\mu$ M) + LPS (100 ng/ml) or IMDM + 10% autologous serum (2.5 h control). Samples were then resolved on a 12 % gel and western blotting carried out according to the protocol in Chapter 2.

The results of this experiment (shown in figure 4.2.7) demonstrate that not only can R-roscovitine override the ability of GM-CSF to promote neutrophil longevity by Mcl-1 but that it can also appears to be able to override the cytoprotective effects of LPS and TNF- $\alpha$  on neutrophils to induce apoptosis. This is a promising find as a novel anti-inflammatory agent must possess the ability to induce neutrophil apoptosis even in the presence of pro-inflammatory agents.



**Key:**

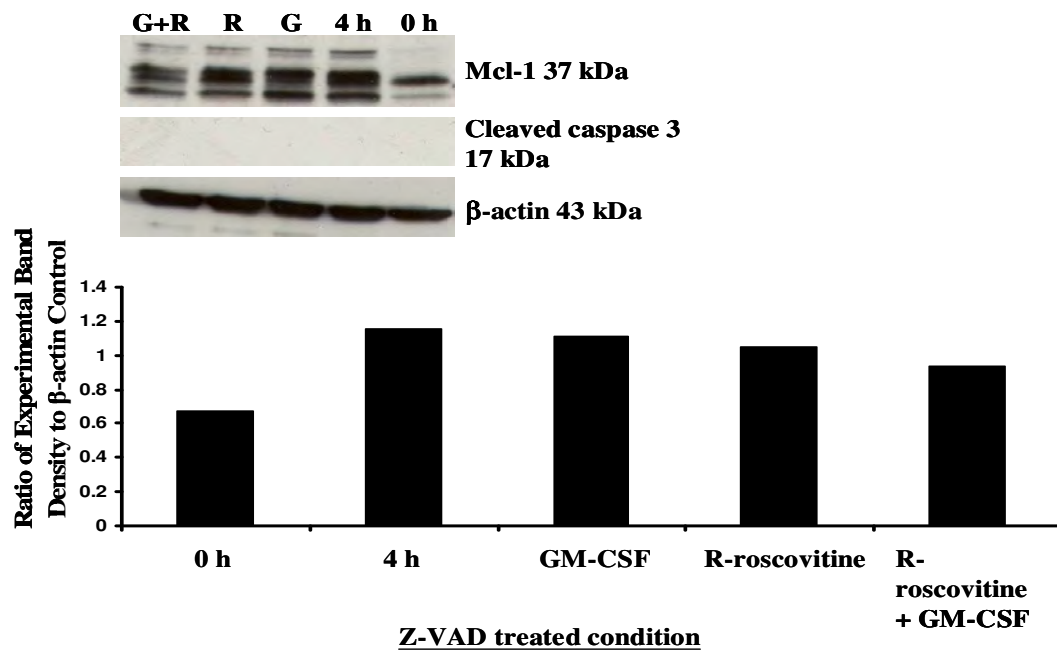
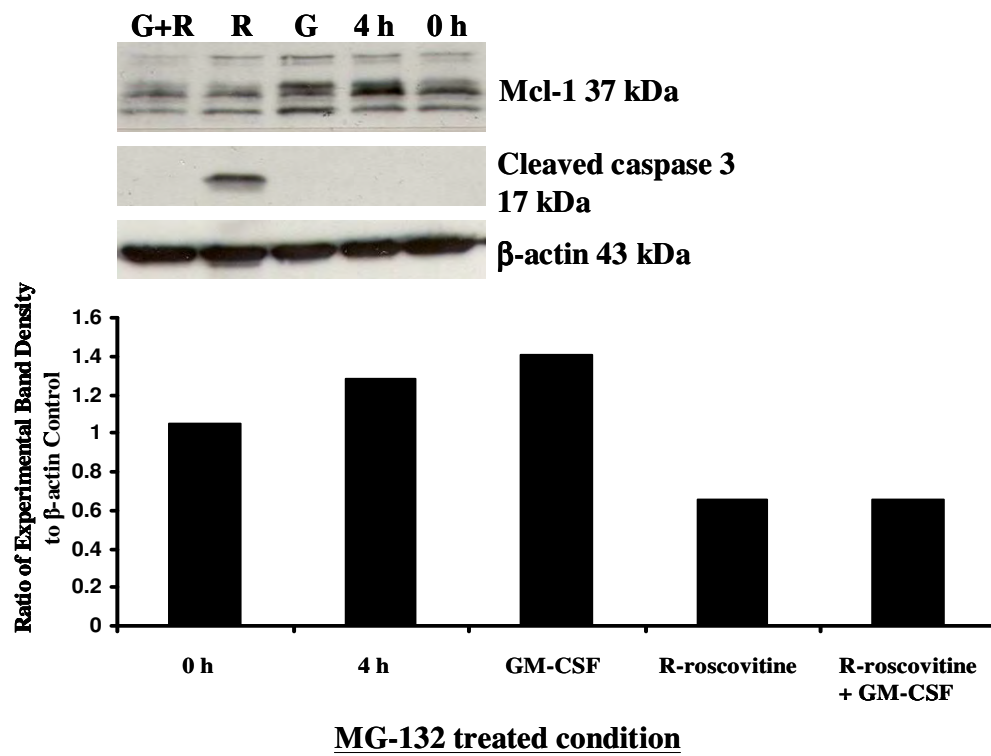
**C = Control, G = GM-CSF, T = TNF- $\alpha$ , L = LPS, R = R-roscovitrine**

**Figure 4.2.7 Effect of R-roscovitrine on GM-CSF, TNF- $\alpha$  and LPS induced neutrophil survival and the anti-apoptotic protein Mcl-1**

*Mcl-1 levels in neutrophils (final concentration  $5 \times 10^6$  /ml) were measured by Western blot analysis immediately after isolation (0 h), or after 2.5 h incubation in a shaking water bath at 37 °C either with GM-CSF (50 ng/ml), TNF- $\alpha$  (10 ng/ml), LPS (100 ng/ml), GM-CSF (50 ng/ml) + R-roscovitrine (20  $\mu$ M), R-roscovitrine (20  $\mu$ M) + TNF- $\alpha$  (10 ng/ml), R-roscovitrine (20  $\mu$ M) + LPS (100 ng/ml) or IMDM + 10 % autologous serum (2.5 h control). The proteins were separated by SDS PAGE, Western Blotted onto PVDF and probed with anti-Mcl-1 antibody (1:200).*

#### **4.2.8 Effect of proteasome and caspase inhibitors on R-roscovitrine and GM-CSF mediation of Mcl-1 in neutrophils**

There is some debate over the method by which the Mcl-1 protein is turned over. Although the protein contains a PEST domain (associated with rapid turn over) deletion of this domain does not affect the stability of Mcl-1 (Akgul et al., 2000a). Alternative methods which could be responsible for the regulation of the Mcl-1 protein are proteolytic degradation by the caspases, or degradation by the proteasome (Derouet et al., 2004, Herrant et al., 2004). Therefore, it was decided to further investigate the post-translational regulation of Mcl-1. Using the global caspase inhibitor Z-VAD-fmk and the proteasome inhibitor MG-132 the effects of caspase and proteasome inhibition on the ability of R-roscovitrine to induced Mcl-1 degradation was investigated. .



Key: G=GM-CSF, R=R-roscovitine

Figure 4.2.8 Effect of proteasome and caspase inhibitors on R-roscovitine and GM-CSF mediation of Mcl-1 in neutrophils

**Figure 4.2.8 Effect of proteasome and caspase inhibitors on R-roscovitine and GM-CSF mediation of Mcl-1 in neutrophils**

*Neutrophils at a final concentration of  $5 \times 10^6$  cells/ml in IMDM + 10 % autologous serum were either lysed immediately (0 h) or treated either with MG-132 (20  $\mu$ M) or Z-VAD (100  $\mu$ M). Conditions were incubated for 4 h at 37  $^{\circ}$ C with gentle agitation with either IMDM + 10 % autologous serum (4 h control), GM-CSF (50 ng/ml), R-roscovitine (20  $\mu$ M) or GM-CSF (50 ng/ml) + R-roscovitine (20  $\mu$ M). Blots were stripped and re-probed with the antibodies against cleaved caspase 3 and  $\beta$ -actin. Histograms represent the ratio of the experimental Mcl-1 bands to the relevant  $\beta$ -actin control, analysed by Image J.*

Our provisional results indicate that inhibition of the proteasome with MG-132 does not affect the anticipated action of R-roscovitine on levels of Mcl-1 (Figure 4.2.8). Furthermore, the presence of cleaved caspase 3 following R-roscovitine treatment indicates that apoptosis has been initiated in the R-roscovitine condition. This implies that the proteasome is not responsible (or is at least redundant) for the modulation of protein levels of Mcl-1 in neutrophils treated with R-roscovitine. Interestingly cleaved caspase 3 does not appear to be present in the GM-CSF + R-roscovitine treatment conditions, implying that competition is occurring between the pro-survival mechanism of GM-CSF and the pro-apoptotic mechanism of R-roscovitine. This is supported by the results obtained with the use of the broad range caspase inhibitor Z-VAD-fmk. In this instance the level of Mcl-1 protein remains approximately constant despite the addition of R-roscovitine. The effectiveness of the caspase inhibitor is evident in the absence of cleaved caspase 3 following R-roscovitine treatment. When considered as a whole this implies that regulation of Mcl-1 protein in neutrophils which have been exposed to the CDK inhibitor R-roscovitine occurs through activation of the caspases and not via the proteasome.

The results of these experiments are reinforced by studies carried out within our group which have demonstrated that R-roscovitine induced neutrophil apoptosis is inhibited by pre-incubation with Z-VAD-fmk (as assessed by flow cytometry). In addition Z-VAD-fmk prevents the ability of R-roscovitine to enhance the resolution of inflammation in

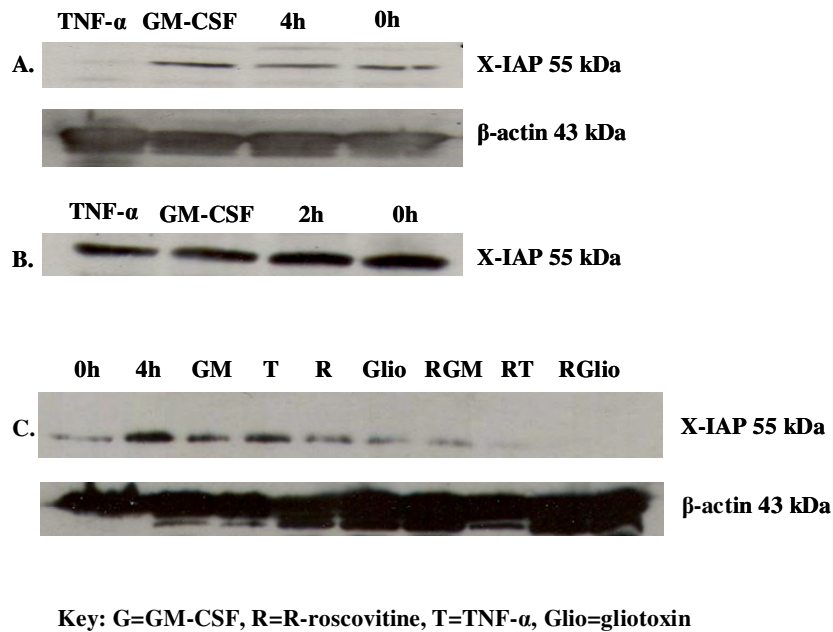
carrageenan-induced pleurisy (Rossi et al., 2006). Taken as a whole, the above results implicate that R-roscovitine is either directly or indirectly regulating the level of Mcl-1 protein through activation of the caspases.

#### **4.2.9 X-IAP levels in neutrophils**

The next stage of our investigation was to look at a survival protein X-IAP and see if some of the effects of R-roscovitine on Mcl-1 were mirrored with this anti-apoptotic protein.

Following the identification of X-IAP protein in neutrophil lysates the effect of R-roscovitine, gliotoxin and the pro-survival cytokines GM-CSF and TNF- $\alpha$  on protein levels was studied. Freshly isolated neutrophils  $5 \times 10^6$  cells/ml in IMDM + 10 % autologous serum were either lysed immediately (0 h) or incubated with either IMDM + 10 % autologous serum (control), 50 ng/ml GM-CSF or 10 ng/ml TNF- $\alpha$  for 4h prior to lysing. Samples were then resolved on a 12% gel and western blotting carried out according to the protocol in Chapter 2. The same protocol was followed only with an incubation time of 2h in Figure 4.2.9 B; and with the additional conditions: 50 ng/ml GM-CSF, 10 ng/ml TNF- $\alpha$ , 20  $\mu$ M R-roscovitine, 0.1  $\mu$ g/ml Gliotoxin, 20  $\mu$ M R-roscovitine + 50 ng/ml GM-CSF, 20  $\mu$ M R-roscovitine + 10 ng/ml TNF- $\alpha$  or 20  $\mu$ M R-roscovitine + 0.1  $\mu$ g/ml gliotoxin for 4h prior to lysing (Figure 4.2.9 C). Blots were stripped and re-probed using an anti-human  $\beta$ -actin antibody, demonstrating equal loading between the conditions.

Representative results are portrayed in Figure 4.2.9, although repetition of findings did prove difficult.



**Figure 4.2.9 Effect of R-roscovitrine on the anti-apoptotic protein X-IAP**

*A) Neutrophils ( $5 \times 10^6$  /ml) were either lysed immediately after isolation (0 h), or after 4 h incubation in a shaking water bath at 37 °C either in IMDM (4 h control), GM-CSF (50 ng/ml), or TNF-α (10 ng/ml). The proteins were separated by SDS PAGE, Western Blotted onto PVDF.*

*B) Follows the same protocol as A) only with an incubation period of 2 h.*

*C) Neutrophils ( $5 \times 10^6$  /ml) were either lysed immediately after isolation (0 h), or after 4 h incubation in a shaking water bath at 37 °C with either IMDM (4 h control), R-roscovitrine (20 μM), GM-CSF (50 ng/ml), TNF-α (10 ng/ml), Gliotoxin (0.1 μg/ml), R-roscovitrine (20 μM) + GM-CSF (50 ng/ml), R-roscovitrine (20 μM) + TNF-α (10 ng/ml), or R-roscovitrine (20 μM) + Gliotoxin (0.1 μg/ml). The proteins were separated by SDS PAGE, Western Blotted onto PVDF.*

Initial experiments suggested that at 4 h TNF- $\alpha$  appeared to have an inhibitory effect on X-IAP expression levels. However, further reproductions of the experiment were not as convincing. Interestingly, it may be possible that R-roscovitine can also override the cytoprotective effects of GM-CSF and TNF- $\alpha$  on X-IAP levels in neutrophils. As can be seen in Figure 4.2.9 C at 4 h there appears to be an increase in expression of X-IAP compared to 0 h. The presence of the cytokines GM-CSF and TNF- $\alpha$  also cause an apparent increase in levels of X-IAP compared to the 0 h control but not to quite the same extent as 4 h alone. In addition R-roscovitine causes a reduction in levels of the X-IAP protein at 4 h reducing it to approximately the same level as was present at 0 h. Therefore, this could indicate that the cell's delicate balance between pro and anti-apoptotic proteins has been shifted and now favours apoptosis.

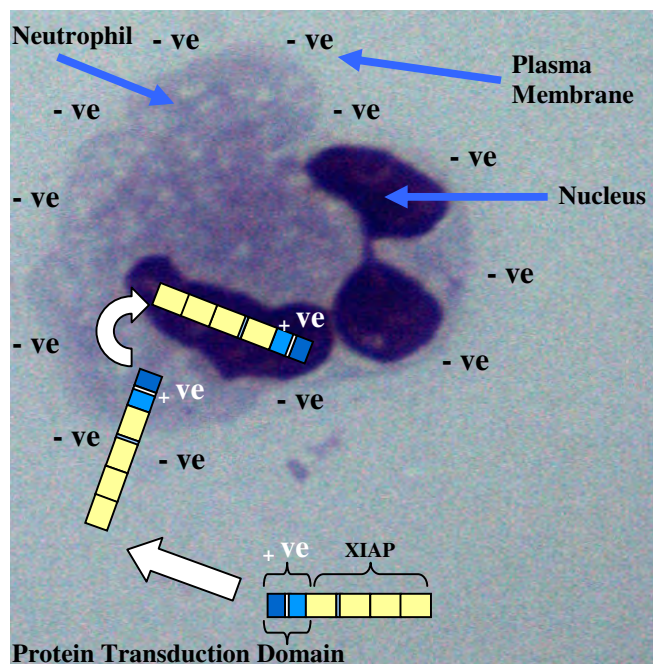
Furthermore, gliotoxin also appears to potentially cause a reduction in X-IAP to that of the 0 h level, which could be due to the inhibition of the transcription factor NF- $\kappa$ B. When R-roscovitine is incubated in conjunction with GM-CSF the level of the X-IAP protein decreases to that of when R-roscovitine is incubated alone, and in addition the level of protein falls even further when incubated with R-roscovitine and TNF- $\alpha$  or R-roscovitine and gliotoxin, with the latter disappearing altogether. This mimics the observations made with Mcl-1 when R-roscovitine was co-incubated with GM-CSF, TNF- $\alpha$  and LPS.

Possible explanations for the inconsistency in results achieved when immunoblotting for X-IAP are that although extensive precautions were taken to ensure that the neutrophil's abundant proteases were inhibited during the experimental process some remained active degrading the proteins. Alternatively, X-IAP is known to be important in the terminal phase of apoptosis therefore the time-points chosen could be confounding the results.

#### 4.2.10 Effects of X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 18 h.

Another approach used in this thesis to investigate further the role of X-IAP was an X-IAP-HIV-tat construct. With this we explored the possibility that X-IAP could block the apoptosis inducing effects of R-roscovitine.

The X-IAP-HIV-tat protein was a kind gift from Dr Brigitte Onteniente. It was generated by fusing the X-IAP protein to the Protein Transduction Domain (PTD) of HIV-1/Tat. The positive charge of the PTD allows the tagged protein to pass through the negatively charged plasma membrane of the neutrophil and the nuclear localization signal located within the tat protein guides the construct into the nucleus where it can affect the function of the cell.



**Figure 4.2.10.1 Diagrammatic representation of X-IAP-tat entering a neutrophil**

*The positive charge of the protein transduction domain attached to the protein of interest allows the protein to passively enter the cell through the negatively charged membrane, where it can then affect the function of the cell. The tat protein also contains a nuclear localization signal allowing the construct to pass into the nucleus.*



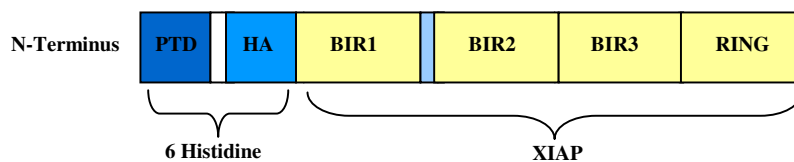
Primers:

5-VGGG CTC GAG ATG ACT TTT AAC AGT TTT GAA GG-3V (sense) and  
5-VGGG GAA TTC TTA AAA CAT AAA AAT TTT TTT GCT TG-3V (antisense)

The above primers were used to isolate the cDNA reading frame of rat X-IAP by PCR. The purified fragments were then cloned into a pPTD-HA vector at the XhoI/EcoRI sites.

#### Final protein encoded:

6-histidine residues tag, PTD (YGRKKRRQRRR), hemagglutinin (HA) tag (YPYDVPDVA), and X-IAP

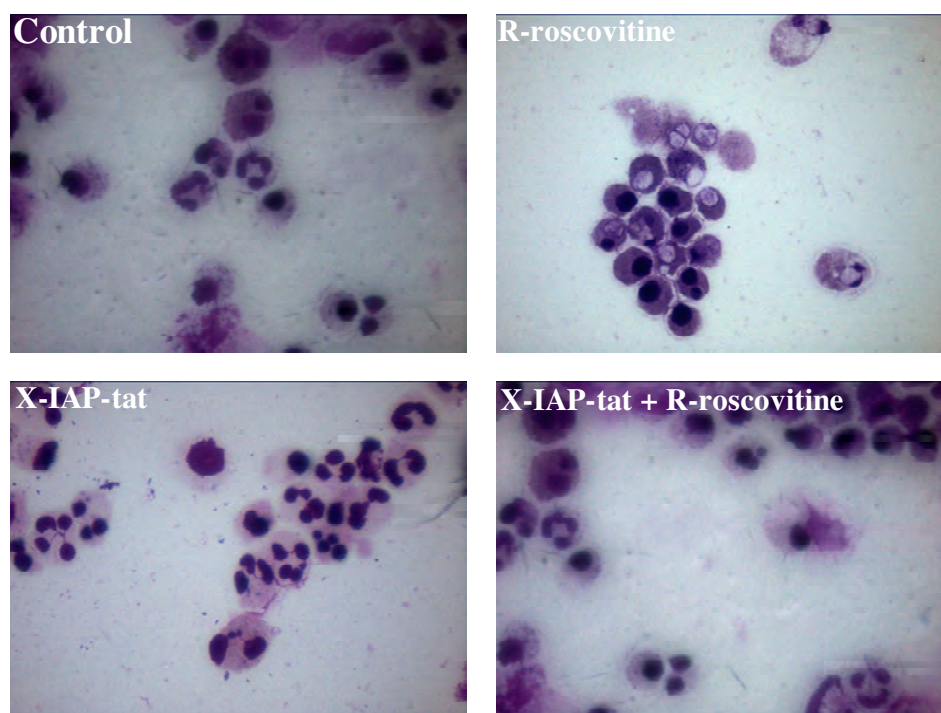


**Figure 4.2.10.2 Diagrammatic representation of the XIAP-tat construct**

*The X-IAP-tat construct contains the 3 BIR domains of the X-IAP protein, along with the RING domain. This is attached to the 6 histidine HIV-tat protein transduction domain at the N-terminus of the protein.*

X-IAP is a 55 kDa protein; the X-IAP-tat construct provided by Dr Brigitte Onteniente was constructed from rat X-IAP. However, as the rat X-IAP protein is 89 % homologous to the human protein we decided to test the effects of the rat X-IAP-tat product on the human neutrophil, specifically in relation to treatment with R-roscovitine.

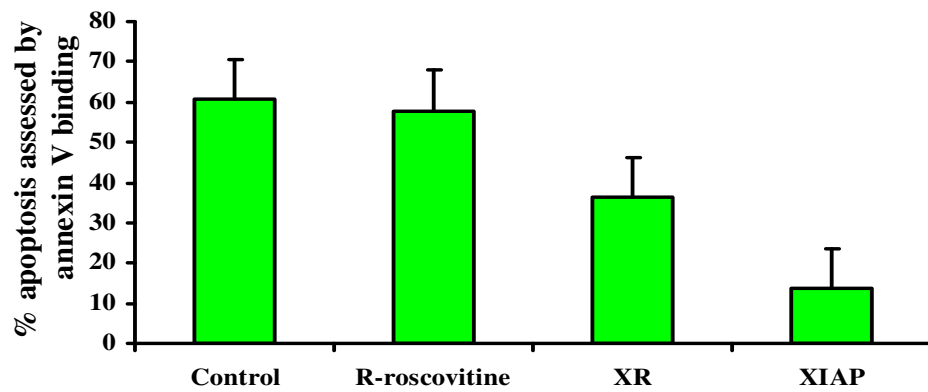
The above HIV-tat X-IAP construct was used to introduce additional X-IAP protein into the cells. Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum in either IMDM (control), 1  $\mu$ M X-IAP-tat, 20  $\mu$ M R-roscovitine or a combination of 1  $\mu$ M X-IAP-tat and 20  $\mu$ M R-roscovitine (XR) and incubated at 37 °C for 18 h before staining with Annexin-V and PI, levels of which were assessed by flow cytometry.



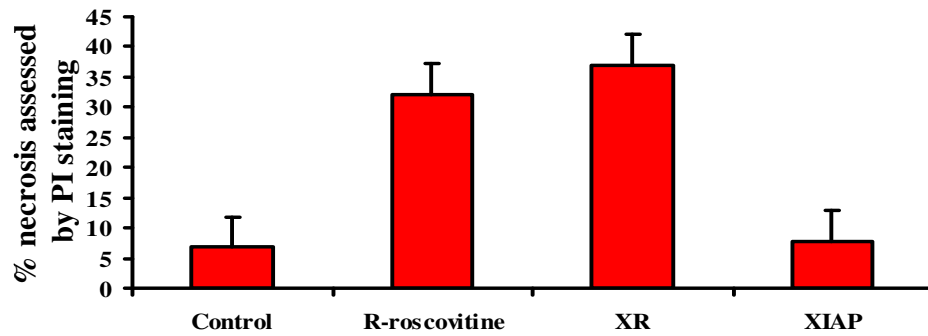
**Figure 4.2.10.3 Photomicrograph (x 400) of the effects of X-IAP-tat and R-roscovitine on neutrophil apoptosis at 18 h**

*Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum with either IMDM (control), 1  $\mu$ M X-IAP-tat, 20  $\mu$ M R-roscovitine or a combination of 1  $\mu$ M X-IAP-tat and 20  $\mu$ M R-roscovitine and incubated at 37 °C for 18 h before staining with eosin and haematoxylin. Visualised by oil immersion microscopy.*

**A.**



**B.**



**Figure 4.2.10.4 Effects of X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 18h.**

*Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum with either IMDM (control), 1  $\mu$ M X-IAP-tat, 20  $\mu$ M R-roscovitine or a combination of 1  $\mu$ M X-IAP-tat and 20  $\mu$ M R-roscovitine (XR) and incubated at 37 °C for 18 h before staining with Annexin-V and PI, levels of which were assessed by flow cytometry. Data shows the results of 3 separate experiments.*

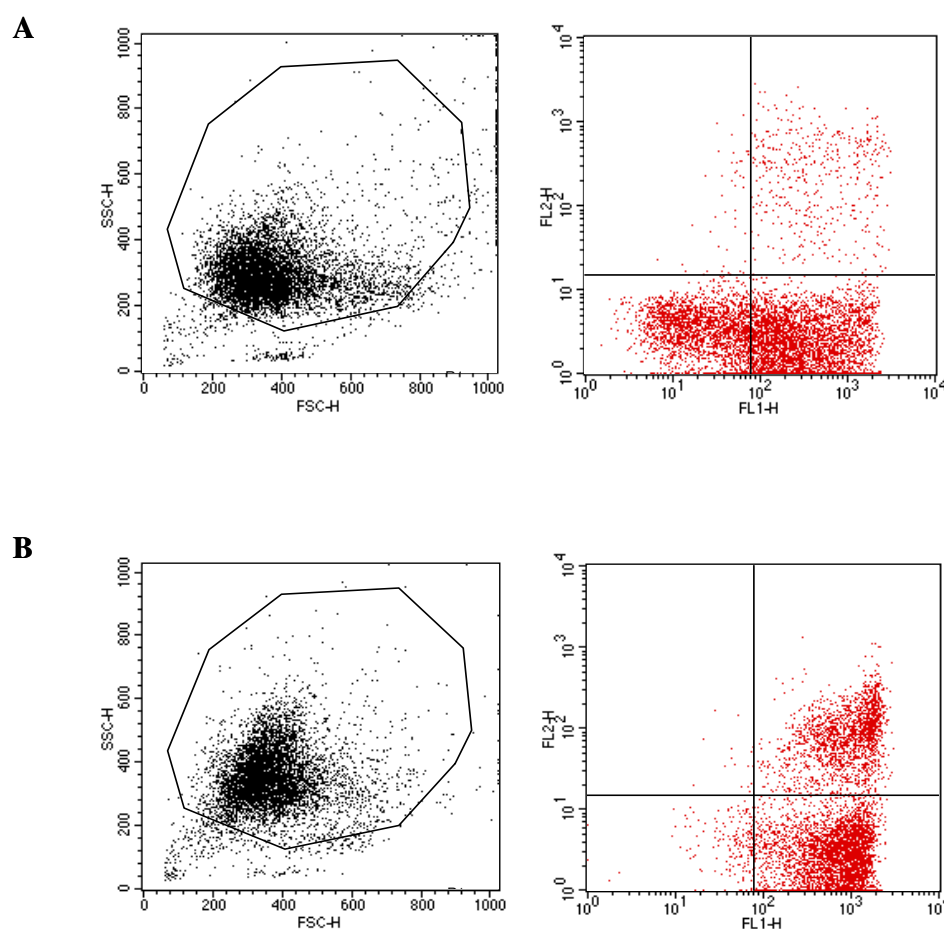
Figure 4.2.10.4 A. shows that Annexin V binding in the control condition at 18 h is ~ 60 %, which is comparable to the ~ 58 % binding obtained when treated with R-roscovitine. However, when the X-IAP-tat construct was combined with R-roscovitine levels of binding reduced to ~ 36 % indicating a reduction in levels of apoptosis, and furthermore when the X-IAP construct was incubated alone, Annexin V binding was ~ 15 %. These results indicate that the X-IAP-tat construct does appear to be having a protective effect on the life-span of the neutrophils and does reduce levels of apoptosis when incubated with R-roscovitine.

However, in order to draw a firm conclusion it is also necessary that PI staining is taken into consideration, which can be seen in figure 4.2.10.4 B. This shows that the control exhibited only ~ 8 % necrosis, compared to ~ 32 % induced by R-roscovitine and ~ 37 % induced by R-roscovitine + X-IAP-tat. This indicates that the cells have passed through apoptosis and into secondary necrosis, which when taken into consideration with the % Annexin V binding indicates that X-IAP-tat does not appear to be causing as great a decrease in cell death as initially thought. However, when X-IAP-tat was incubated alone it does appear to have a cyto-protective effect on neutrophils as levels of necrosis are also low at ~ 8 %. When percentage apoptosis and necrosis are combined neutrophil death in the control is ~ 67 %, in the R-roscovitine treated condition ~ 90 %, which is reduced to 73 % when R-roscovitine is co-administered with X-IAP-tat and when neutrophils were incubated with X-IAP-tat alone cell death is decreased to only 22 %. Taken as a whole this appears to indicate that the X-IAP-tat construct is having a cyto-protective effect on the neutrophils, which can partially overcome R-roscovitine induced apoptosis.

#### **4.2.11 Shape change in neutrophils treated with X-IAP-tat at 18 h.**

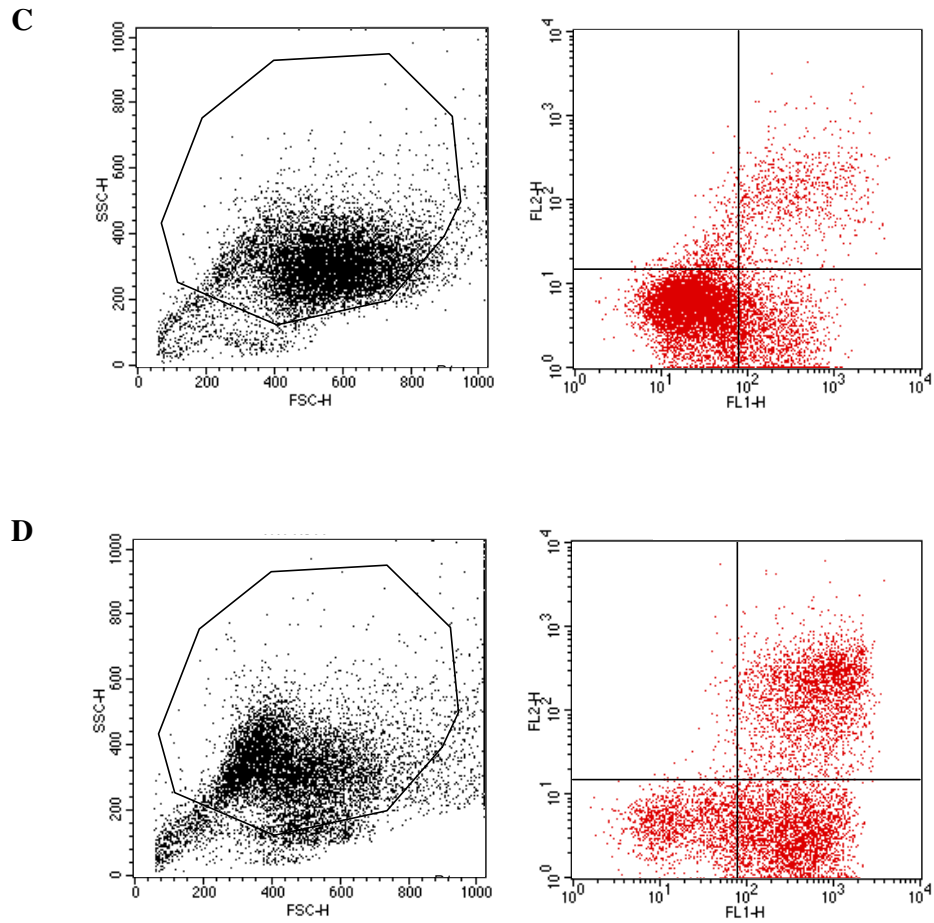
The analyses of the previous experiment indicated that there appeared to be a change in forward scatter. This is demonstrated via a shift to the right in forward-scatter in conditions where the X-IAP-tat construct was used, as can be seen in Figure 4.2.11 C (X-IAP-tat) where the median of the cells lies at ~ 600 FSC-H and D (X-IAP-tat + R-roscovitine) where the median lies at ~ 500 FSC-H compared to Figure 4.2.11 A (control) and B (R-

roscovitine) which both lie at  $\sim 350$  FSC-H. This implies that either the tat construct is having an effect similar to priming and encouraging the neutrophils to spread. Alternatively this could indicate LPS contamination of the X-IAP-tat construct from the bacterial system which it was derived. This is because LPS causes priming of neutrophils, visualised by a change in shape and shift in forward scatter. Because of this observation the next step taken was to compare the effects of LPS to those of the tat construct.



**Figure 4.2.11.1** Flow cytometric dot plots showing forward scatter versus side scatter and FL1 versus FL2 of neutrophils aged for 18 h with R-roscovitine

*Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum in either the absence (A) control or presence of 20  $\mu$ M R-roscovitine (B) for 18 h before staining with Annexin-V and PI, levels of which were assessed by flow cytometry. Data shows the results of 3 separate experiments.*



**Figure 4.2.11.2 Flow Cytometric Dot plots showing forward scatter versus side scatter and FL1 versus FL2 of neutrophils aged for 18 h with either X-IAP-tat or X-IAP-tat + R-roscovitine**

*Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum in either the presence of  $1 \mu$  M XIAP-TAT (C) or  $1 \mu$  M XIAP-TAT +  $20 \mu$  M R-roscovitine (D) for 18 h before staining with annexin-V and PI, levels of which were assessed by flow cytometry. Data shows the results of 3 separate experiments.*

#### 4.2.12 Effects of X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 4 h.

To further investigate the effects of the X-IAP-tat construct on R-roscovitine induced apoptosis and possible LPS contamination an LPS treated condition was introduced to allow comparison with the control and tat treated conditions. In addition a new time point of 4 h was chosen as the previous 18 h time point was of such duration that the neutrophils had passed through apoptosis and started to enter secondary necrosis. A time point of 4 h prevents this whilst still being adequate for the apoptosis inducing effects of R-roscovitine to be observed.

Figure A. shows the Annexin V binding and PI staining of neutrophils ( $10 \times 10^6$  /ml) treated in IMDM + 10 % autologous serum with either IMDM (control), 1  $\mu$ M X-IAP-TAT, or 100 ng/ml LPS for 4 h plus either IMDM (control) or 20  $\mu$ M R-roscovitine after the first h for the remaining 3 h. Samples were incubated at 37 °C throughout the time point. The control/control condition demonstrates ~ 1.25 % apoptosis (assessed by Annexin V binding) compared to ~ 3 % when R-roscovitine is present (control/R-roscovitine), demonstrating that even at this early time-point R-roscovitine has the ability to induce apoptosis in neutrophils. The ability of LPS to inhibit neutrophil apoptosis is evident by the % apoptosis reducing to ~ 0.75 % and the presence of R-roscovitine with LPS slightly increases this to ~ 0.9 %. The X-IAP-tat construct alone at this time point does not alter the percentage apoptosis when compared to the control (~ 1.2 % to ~ 1.25 % respectively) which is to be expected as at this time point the neutrophils will not have entered spontaneous apoptosis. However, when R-roscovitine is incubated in conjunction with X-IAP-tat, the tat construct reduces the ability of R-roscovitine to induce apoptosis from ~ 3 % when incubated alone to ~ 1.5 % when X-IAP-tat is present. This does again initially indicate that X-IAP-tat is having a cyto-protective effect on R-roscovitine induced neutrophil apoptosis. Interestingly, LPS appeared to inhibit apoptosis both when alone and when in conjunction with R-roscovitine.

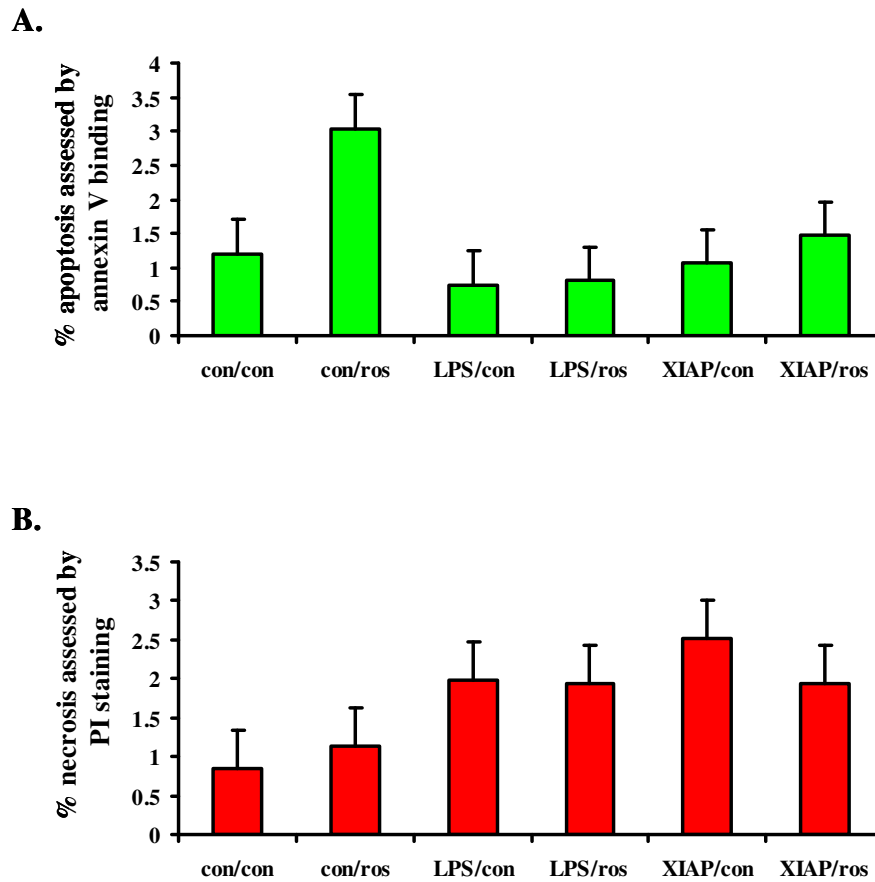
When PI staining is examined (Figure B.) it can be seen that at 4 h the percentage necrosis for the control is low at ~ 0.75 %, this is similar to the level obtained in the control/R-

roscovitine condition (~ 1.2 %). However, when compared to the control LPS, LPS + R-roscovitine, X-IAP and X-IAP + R-roscovitine all cause a slight increase in necrosis at ~ 2 %, ~ 1.9 %, ~ 2.5 % and ~ 1.9 % respectively. Given that the results obtained with LPS, LPS + R-roscovitine, X-IAP and X-IAP + R-roscovitine were all fairly similar the flow cytometric dot-plots and percentage shape change (a known indicator of priming/activation of neutrophils) were examined.

#### **4.2.13 Shape change in neutrophils treated with X-IAP-tat at 4 h.**

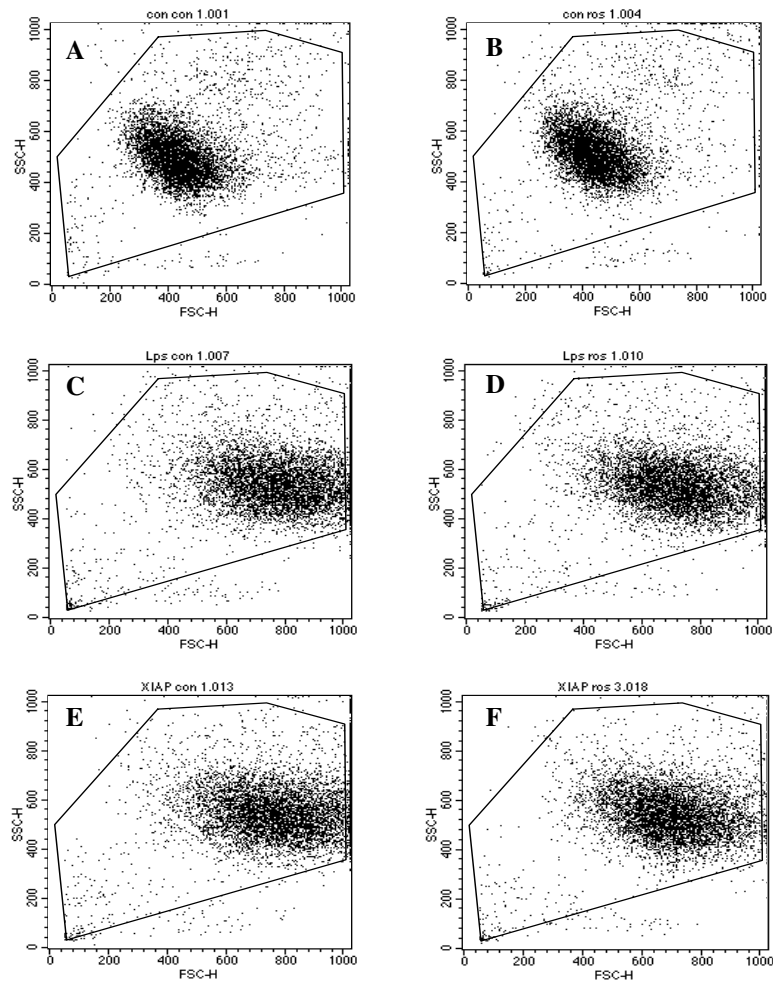
The change in forward scatter visualised in the flow cytometric dot plots at 4 h indicates a distinct shift in the populations to the right when the neutrophils were incubated with LPS, LPS + R-roscovitine, X-IAP and X-IAP + R-roscovitine (Figure 4.2.13.1). This was examined more objectively through adding a gate on the forward scatter histogram which allows the percentage shape change to be examined by comparison of all values to the control (Figure 4.2.13.2). When presented as a bar chart it can be seen that both the control and control + R-roscovitine cause approximately 10 % shape change whilst LPS, LPS + R-Roscovitine, X-IAP and X-IAP + R-roscovitine cause approximately 90 %, 85 %, 87 % and 80 % shape change respectively. This indicates that there is something either intrinsic to or contaminating the X-IAP-tat construct and causing priming/activation of the neutrophils that is similar to LPS.





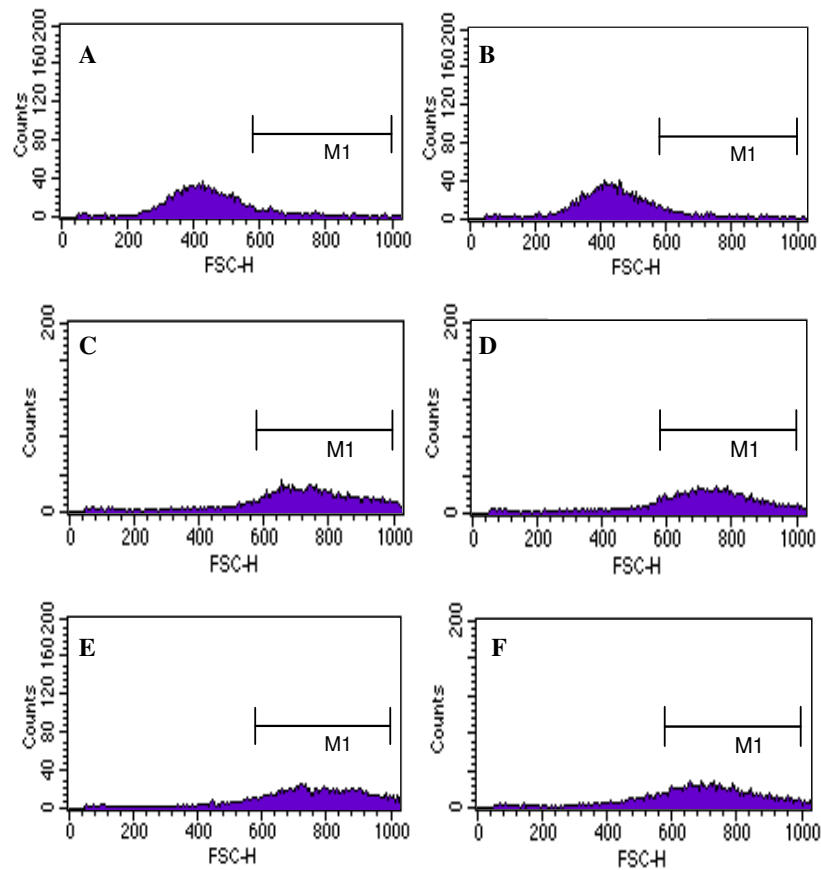
**Figure 4.2.12 Effects of the X-IAP-tat construct and R-roscovitine on neutrophil apoptosis at 4 h in the presence or absence of LPS.**

*Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum as follows. Control/Control in the absence of any agents for 4 h. Control/R-roscovitine in the absence of any agents for 1 h and then 20  $\mu$ M R-roscovitine added for 3 h. LPS/Control treated with 100 ng/ml LPS for 4 h with media added after 1 h for the remaining 3 h. LPS/R-roscovitine treated with 100 ng/ml LPS for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. X-IAP/Control treated with 1  $\mu$ M X-IAP-TAT for 4 h with media added after 1 h for the remaining 3 h. X-IAP/R-roscovitine treated with 1  $\mu$ M X-IAP-TAT for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. Samples were then stained with Annexin-V (A) and PI (B) levels of which were assessed by flow cytometry. Data representative of 3 separate experiments.*



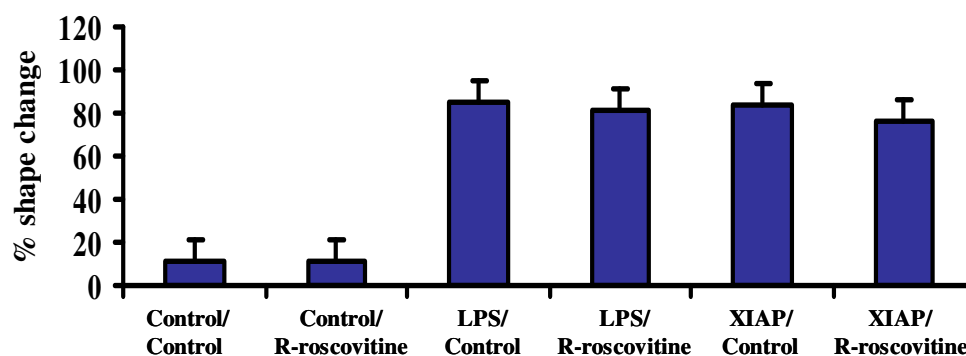
**Figure 4.2.13.1** Flow Cytometric Dot Plots showing forward scatter verses side scatter of neutrophils aged for 4 h with X-IAP-tat or R-roscovitine in the presence or absence of LPS.

*Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum as follows. (A) in the absence of any agents for 4 h. (B) in the absence of any agents for 1 h and then 20  $\mu$ M R-roscovitine added for 3 h. (C) treated with 100 ng/ml LPS for 4 h with media added after 1 h for the remaining 3 h. (D) treated with 100 ng/ml LPS for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. (E) treated with 1  $\mu$ M X-IAP-TAT for 4 h with media added after 1 h for the remaining 3 h. (F) treated with 1  $\mu$ M XIAP-TAT for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. Samples were then stained with Annexin-V and PI levels of which were assessed by flow cytometry. Data representative of 3 separate experiments.*



**Figure 4.2.13.2** Flow Cytometric histograms showing shape change induced in neutrophils aged for 4 h with X-IAP-tat or R-roscovitine in the presence or absence of LPS.

*Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum as follows. (A) in the absence of any agents for 4 h. (B) in the absence of any agents for 1 h and then 20  $\mu$ M R-roscovitine added for 3 h. (C) treated with 100 ng/ml LPS for 4 h with media added after 1 h for the remaining 3 h. (D) treated with 100 ng/ml LPS for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. (E) treated with 1  $\mu$ M X-IAP-TAT for 4 h with media added after 1 h for the remaining 3 h. (F) treated with 1  $\mu$ M X-IAP-TAT for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. Samples were then stained with Annexin-V and PI levels of which were assessed by flow cytometry. Data representative of 3 separate experiments.*



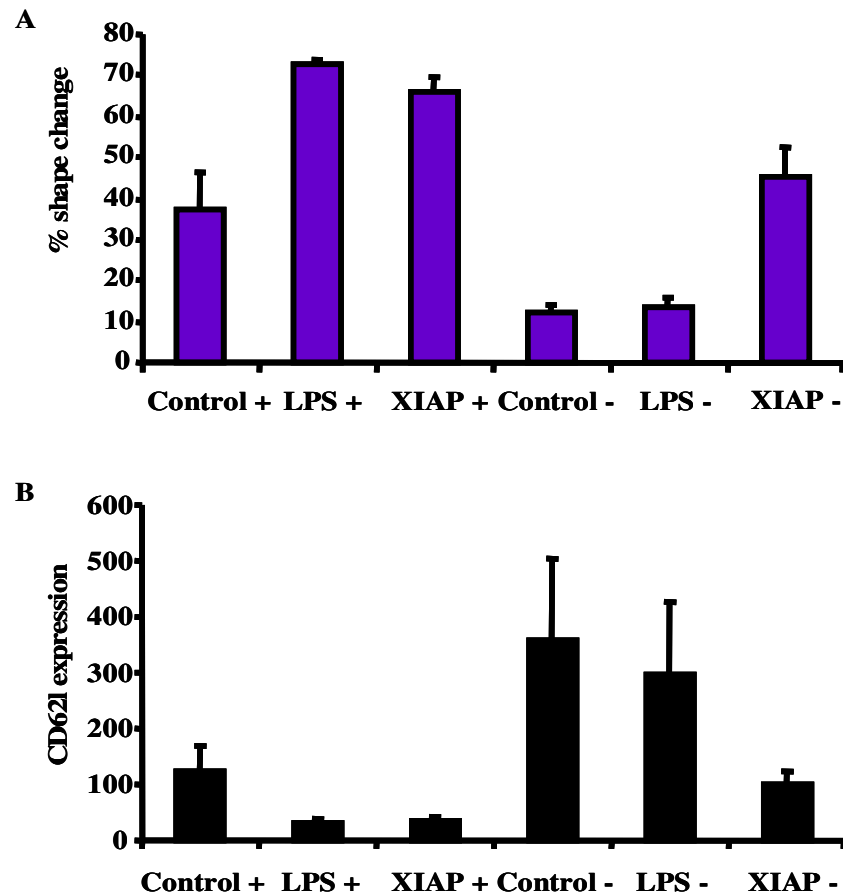
**Figure 4.2.13.3** Histograms showing percentage shape change for neutrophils aged for 4 h with X-IAP-tat or R-roscovitine in the presence or absence of LPS.

*Neutrophils ( $10 \times 10^6$  /ml) were treated in IMDM + 10 % autologous serum as follows. Control/Control in the absence of any agents for 4 h. Control/R-roscovitine in the absence of any agents for 1 h and then 20  $\mu$ M R-roscovitine added for 3 h. LPS/Control treated with 100 ng/ml LPS for 4 h with media added after 1 h for the remaining 3 h. LPS/R-roscovitine treated with 100 ng/ml LPS for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. X-IAP/Control treated with 1  $\mu$ M XIAP-TAT for 4 h with media added after 1 h for the remaining 3 h. X-IAP/R-roscovitine treated with 1  $\mu$ M X-IAP-TAT for 4 h with 20  $\mu$ M R-roscovitine added after 1 h for the remaining 3 h. Samples were then stained with Annexin-V and PI levels of which were assessed by flow cytometry. Data representative of 3 separate experiments.*

#### **4.2.14 Effect of X-IAP-tat on CD62L expression and shape change in neutrophils.**

Because of the similarity of the effects of X-IAP-tat and LPS on neutrophil shape change their effects on the selectin CD62L, which is shed upon neutrophil activation, were investigated (Figure 4.2.14). Neutrophils were resuspended at  $5 \times 10^6$  /ml in PBS + cations. Half of the samples were treated with media that contained 10 % autologous serum (+) and half were treated without serum (-). Within each of these two conditions one sample was treated without any additional reagents (control) one was treated with 100 ng/ml LPS and one with 1  $\mu$ M X-IAP-tat for 30 min prior to incubation with anti-human anti-CD62L for

30 min. Samples were then analysed using flow cytometry. Samples were treated in either the presence or absence of serum as the serum factors LBP, CD14 and MD2 are known to be required for LPS to bind to and activate LPS signaling through TLR4.



**Figure 4.2.14** Histograms showing collated data for neutrophil shape change and CD62L expression induced by X-IAP-tat and LPS either in the presence or absence of autologous serum.

*Neutrophils were resuspended at  $5 \times 10^6$  /ml in PBS + cations. Half of the samples were treated with media that contained 10 % autologous serum (+) and half were treated without serum (-). Within each of these two conditions one sample was treated without any additional reagents (control) one was treated with 100 ng/ml LPS and one with 1  $\mu$ M XIAP-TAT for 30 min prior to incubation with anti-human anti-CD62L for 30 min. Samples were then analyzed using flow cytometry. Data representative of 3 separate experiments.*

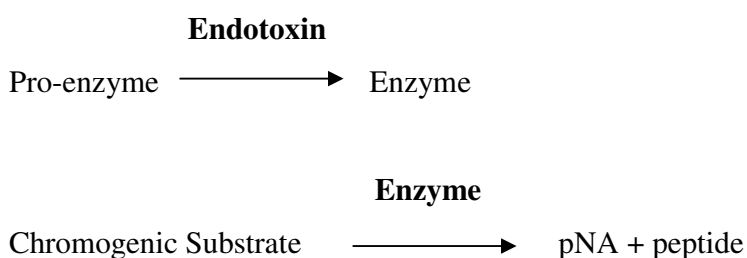
Confirmation of the previous shape change results were observed as demonstrated in figure 4.2.14 A. where in the presence of 10 % autologous serum LPS and X-IAP-tat caused an increase in percentage shape change of approximately 34 % and 30 % respectively when compared to the serum control. However, in the absence of 10 % autologous serum LPS only causes ~ 2 % increase in percentage shape change compared to the serum free control. Whereas, the X-IAP-tat construct causes ~ 34 % increase. This confirms that in order for LPS to activate its signaling cascade in neutrophils the serum binding factors must be present. In addition the X-IAP-tat construct when in the absence of serum still results in shape change, albeit to a lesser extent than in the presence of serum. This indicates that the X-IAP-tat construct itself may be responsible for the shape change, or possibly that at some point during its construction it was contaminated by LPS and its binding proteins.

In addition when CD62L was investigated (Figure 4.2.14 B) it appears that the presence of serum alone affects the rate of CD62L shedding in neutrophils with there being a difference in geo mean fluorescence of 225 between the control with and the control without serum. However, in both cases LPS and X-IAP-tat result in a decrease in expression of CD62L, although when serum is present the decrease caused by LPS and X-IAP-tat is very close with both resulting in a reduction of ~ 90. Whereas when serum is absent the effect of LPS on CD62L shedding is markedly reduced from the serum negative control value of ~ 350 to ~ 300 compared to ~ 100 in the serum absent X-IAP-tat construct condition. Again as the X-IAP-tat construct causes shape change and CD62L shedding it can be concluded that it is having a priming effect on the neutrophil. In addition the similarity between the effects of the X-IAP-tat construct and LPS in the serum containing conditions could indicate that the possible cause of the X-IAP-tat constructs ability to prime/activate neutrophils could be that it is contaminated by LPS and its binding proteins as it still has the power to activate CD62L shedding in the absence of serum.

#### 4.2.15 Limulus amoebocyte lysate (LAL) test of the X-IAP-tat construct

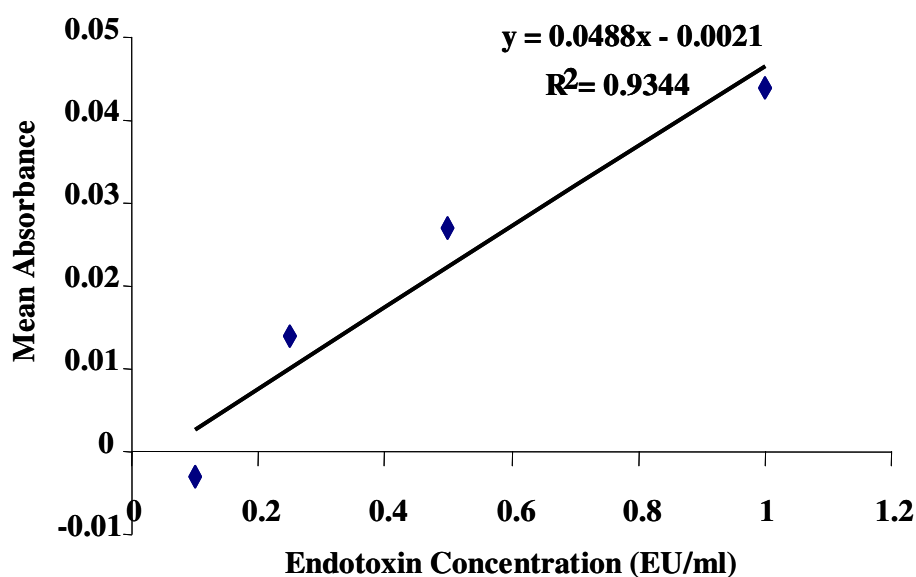
To ascertain conclusively whether the X-IAP-tat construct was contaminated with LPS a LAL test was performed.

The LAL assay was developed from the observation of Frederick Bang that intravascular coagulation was caused by bacteria in *Limulus polyphemus* (American horseshoe crab). Levin and Bang concluded that this was caused by the bacterial endotoxin causing an enzymatic reaction in the amoebocytes. These enzymatic reactions form the basis of the LAL assay, upon enzymatic cleavage of the chromophore p-nitroaniline (pNA) the colourless substrate will become yellow; this reaction is terminated by the addition of acetic acid. The levels of pNA created are proportional to the amount of endotoxin present and the colour change is measured at 450 nm with a spectrophotometer. The standard samples can be used to create a standard curve from which the endotoxin concentration of the experimental conditions is calculated.



The results from this indicate that the X-IAP-tat construct contained contaminating LPS at approximately 45.72µg/ml, a significant amount compared to the 100ng/ml throughout this thesis. This therefore offers an explanation as to why results obtained in the previous experiments were similar to that of LPS. However, it can not be ruled out that the pro-survival effects of the X-IAP-tat construct were not, at least in part, due to transduction of exogenous X-IAP into the neutrophil, inhibiting apoptosis and therefore prolonging the lifespan of the cell. If time constraints were not present the X-IAP-tat construct could be tagged with green fluorescent protein to confirm whether the construct is actually entering

the cell, and the proteins localisation. Columns could also be purify the construct and remove any contaminating LPS.



Samples diluted 1 in 10	Readings	Absorbance	Concentration (µg/ml)
XIAP tat	0.31	0.223	45.72

**Figure 4.2.15** Standard curve calculated from serial dilutions of standards used to calculate the endotoxin concentration of the XIAP-tat construct.

*The LAL standard curve indicates that the X-IAP-tat construct was contaminated with 45.72 µg/ml of LPS.*



### 4.3 Discussion

The mechanism through which R-roscovitine induces apoptosis in neutrophils has yet to be fully elucidated. Discovery of this would enable the development of novel CDKis which could be honed for treatment of inflammation, targeting specific pathways to induce apoptosis in neutrophils, whilst having minimal negative impact on the functions of the surrounding cells. In this chapter we aimed to shed light on the potential mechanism(s) of action of R-roscovitine in the terminally differentiated neutrophil, which do not require CDKs for the traditional function of cell cycle regulation.

Firstly we chose to study the effect of R-roscovitine on the transcription factor NF- $\kappa$ B. NF- $\kappa$ B was chosen as it is already known to be a key component in controlling the fate of the neutrophil (Ward et al., 1999a), upregulating transcription of anti-apoptotic genes such as the inhibitor of apoptosis protein family (IAP) (Gyrd-Hansen and Meier, 2010). Therefore, if R-roscovitine blocked NF- $\kappa$ B activation it would prevent transcription of anti-apoptotic genes helping to tip the delicate balance between the pro and anti-apoptotic proteins towards apoptosis. NF- $\kappa$ B activation was assessed by I $\kappa$ B $\alpha$  degradation as well as by following the translocation of the p65 subunit. We chose these two approaches as it has been previously reported that NF- $\kappa$ B activation can sometimes occur in the absence of I $\kappa$ B $\alpha$  degradation (Jiménez et al., 2000). Whilst analysis of the confocal micrographs following the translocation of p65 were performed in a semi-blind manner (i.e. the slide labels were not intentionally looked at before viewing) the power of the results could have been increased by performing a full blind analysis ensuring the labels were fully covered and the order in which they were viewed not revealed until the results had been collated.

Our results demonstrate that R-roscovitine does not directly activate NF- $\kappa$ B and furthermore it does not prevent activation of NF- $\kappa$ B by LPS or by TNF- $\alpha$ . From this it can be concluded that R-roscovitine is not inducing apoptosis via a pathway that directly involves early NF- $\kappa$ B activation.

These findings challenge Dai's hypothetical model that CDK inhibitors such as flavopiridol cause inactivation of the NF- $\kappa$ B pathway leading to JNK activation and inhibition of X-IAP expression and therefore apoptosis (Dai et al., 2003). However, R-roscovitine may work through a different NF- $\kappa$ B independent pathway to flavopiridol, or differences between these findings could possibly be explained by variations in the intrinsic pathways of U937 cells and neutrophils. Having said this, even though NF- $\kappa$ B has been ruled out as a contender through which R-roscovitine directly signals in neutrophils it may still become activated through further downstream events. In addition results obtained from the experiments into the effects of R-roscovitine on X-IAP levels in neutrophils were variable (discussed in greater detail later in the chapter).

The next stage of our investigation was to explore the effect of R-roscovitine on the anti-apoptotic proteins Mcl-1 and X-IAP. These were chosen as previous literature has identified them as being key players in regulating the fate of neutrophils (Edwards et al., 2004, Gardai et al., 2004, Hahntow et al., 2004). In addition X-IAP, unlike Mcl-1, is under regulation by NF- $\kappa$ B therefore allowing the effect of R-roscovitine to be examined on two anti apoptotic proteins that are under the regulation of different transcription factors (Gyrd-Hansen and Meier, 2010, Akgul et al., 2000b).

An observation was made whilst Western Blotting for Mcl-1 (Figure 4.2.5) in the neutrophil of two additional bands at a slightly lower molecular weight. Possible explanations for these are: they are post translational modifications of the Mcl-1 protein; alternatively even though every effort was made to inhibit the vast arsenal of neutrophil proteases some break down of the Mcl-1 protein may have occurred; or that the antibody is detecting Mcl-1 short as well as Mcl-1 long. The latter proposition is supported by the additional band running at a similar molecular weight to that of Mcl-1 short, approximately 28 kDa.

From the results obtained in this chapter it is evident that R-roscovitine reduces levels of Mcl-1 allowing the neutrophils to enter apoptosis, this is made even more fascinating as it has been shown that Mcl-1 is essential for the survival of neutrophils playing a non-

redundant role in the regulation of neutrophil apoptosis (Dzhagalov et al., 2007) which is of particular interest in light of our findings as decreases in Mcl-1 levels caused by R-roscovitine may not be compensated by the other survival proteins present in neutrophils resulting in the cell entering apoptosis. In addition R-roscovitine appears to be inducing apoptosis in neutrophils by the caspases, as inhibition with the broad spectrum caspase inhibitor zVAD-fmk prevented R-roscovitine induced reduction of Mcl-1.

We also found that R-roscovitine has the ability to override the protective effect of GM-CSF on Mcl-1 levels at 4h. Interestingly, this phenomenon does not seem to be exclusive to GM-CSF, as when R-roscovitine is used in conjunction with other inflammatory mediators, for example LPS, levels of Mcl-1 are also reduced (the same is potentially true for X-IAP; however, further research is required before this can be stated with confidence). This is a promising finding as it indicates that R-roscovitine can potentially counteract the anti-apoptotic effects of inflammatory mediators at sites of inflammation. When examining the effects of R-roscovitine on X-IAP via Western Blotting the observation was made that the combined effect of R-roscovitine plus the NF- $\kappa$ B inhibitor gliotoxin results in maximal X-IAP reduction. If this is true it would fall in line with Dai's finding that disruption of NF- $\kappa$ B potentiates the apoptotic effect of the CDK inhibitors implying that NF- $\kappa$ B does have a role, albeit not a direct one, in apoptosis induction in neutrophils (Dai et al., 2003).

A possible target for R-roscovitine is the PI3-K/Akt pathway and it has been demonstrated in the human erythroleukaemia cell line Tf-1 that the Mcl-1 gene is upregulated through this pathway (Huang et al., 2000). Therefore, one possible explanation for the decrease in Mcl-1 levels in neutrophils treated with R-roscovitine is that R-roscovitine could be blocking the PI3-K / Akt pathway. However, it has been previously published that in human leukaemic cell lines such as the U937 monocytic leukaemic cell line, CDK's ability to induce apoptosis can be enhanced when combined with PI3-K inhibitors, where they observed decreases in Mcl-1 and X-IAP, which implies only a partial inhibition of the PI3-K/Akt pathway by R-roscovitine. This work was primarily carried out on a monocytic leukaemic cell lines which may possess different regulatory mechanisms to those of neutrophils. However, it does state that similar results have been demonstrated in HL60

cells which can be used as a model for neutrophils, although several intrinsic differences between HL60 cells and primary neutrophils have been reported making extrapolation of results tenuous in some instances (Yu et al., 2003).

Alternatively R-roscovitine could be influencing the ERK pathway, as R-roscovitine has an inhibitory effect on both the ERK1 and ERK2 kinases, although in these instances R-roscovitine was used at a higher concentration than in this thesis (Knockaert et al., 2002, Bach et al., 2005). Interestingly, the ERK 1/2 pathway has been implicated in the resolution of inflammation in rat carrageenan-induced pleurisy, as inhibition of ERK1/2 kinases by PD98059 produced enhanced resolution of inflammation. The proposed mechanism for this is that PD98059 prevents activation of ERK by inflammatory mediators which induce neutrophil survival (Sawatzky et al., 2006). Given more time this would be investigated through the use of an ERK inhibitor such as PD98059 to elucidate if similar effects are achieved to those exhibited by R-roscovitine, and if combined administration alters the anticipated response of the survival proteins. However, it has since been demonstrated in our laboratory (Leitch et al., 2010) that, unlike in vascular smooth muscle cells (Li et al., 2008), R-roscovitine only has a slight effect on LPS induced ERK phosphorylation, whereas PD98059 caused much greater inhibition. This, when considered in conjunction with Figures 4.2.2 and 4.2.3 which demonstrates that R-roscovitine does not inhibit LPS and TNF- $\alpha$  induced activation of NF- $\kappa$ B, implies that R-roscovitine is overriding the anti-apoptotic outcome normally produced by these pathways down-stream of these occurrences, and that the presence of R-roscovitine is significant enough to overcome them.

Whilst it is possible that the above could potentially indicate R-roscovitine induces apoptosis via more than one pathway (as supported by the observation that it can interfere with various inflammatory mediators' ability to prolong neutrophil survival by effects on Mcl-1) it is also plausible that the dramatic induction of neutrophil apoptosis by R-roscovitine could reflect a more universal effect is being imposed. For example, if transcription is compromised by inhibition of CDK7 and CDK9, levels of the subsequent protein will decline (MacCallum et al., 2005). This effect will be particularly prominent in

proteins which have short half lives such as Mcl-1. Interestingly X-IAP has a considerably longer half life than Mcl-1 offering a possible explanation to the lack of conclusive results obtained when immunoblotting for the X-IAP protein.

Unfortunately whilst results from the transduction of X-IAP into neutrophils by the use of a HIV-tat vector were confounded by the discovery of LPS, a relic of the E-coli production vector, the initial administration of the X-IAP-tat construct did appear to have some cytoprotective effect on neutrophils exposed to R-roscovitine, which could be partially due to successful transduction. However, the results could have been further confounded just by the presence of R-roscovitine. This is because it has recently been discovered that CDKis such as R-roscovitine prevent trans-activation of HIV-tat proteins as they inhibit CDK9, an essential mediator of this function in conjunction with its regulatory partner T1 (Canduri et al., 2008).

For conclusive results to be obtained in the presence of CDKis an alternative method of protein transduction is required. Possible methods include microinjection, complexing the X-IAP protein with a lipid or use of virus-like particles which assemble into nanoparticles but lack the ability to replicate. Furthermore tagging of the X-IAP protein construct with GFP would enable the localisation of the introduced protein to be determined. A recent paper by Sabroe *et al.* has shown effective delivery of proteins into primary human neutrophils by lentivirus vectors (Dick et al., 2009). Use of a lentivirus to transduce Mcl-1 and X-IAP proteins into primary human neutrophils treated with R-roscovitine would provide a valuable model to further investigate the roles of these proteins in R-roscovitine induced neutrophil apoptosis.

In conclusion, the results from this chapter indicate that loss of Mcl-1 induced by R-roscovitine is a newly identified mechanism of inducing neutrophil apoptosis. This mechanism appears to be regulated by the caspases, demonstrated through use of the zVAD-fmk and by R-roscovitine induced cleavage of caspase-3. Interestingly when co-incubated with GM-CSF, R-roscovitine loses its ability to induce caspase-3 cleavage. A possible explanation for this is competition between the pro- and anti-apoptotic pathways.

Although a reduction in X-IAP was observed when neutrophils were incubated with R-roscovitine, results were inconsistent and therefore no conclusive theory can be arrived at. This could be, at least in part, due to X-IAP being involved in the concluding stages of apoptosis. As results from the investigations into X-IAP were variable we feel that whilst X-IAP is undoubtedly implicated in neutrophil apoptosis it is possible that the reduction of X-IAP observed is as a result of the neutrophil already being committed to apoptosis and does not share the same causal involvement as Mcl-1.

## **Chapter 5**

### **5.1 Summary and General Discussion**

The inflammatory response is an evolutionary conserved, tightly regulated series of inter-related cellular processes, resolution of which is dependent upon effective removal of apoptotic cells by professional phagocytes such as macrophages. There is mounting evidence suggesting that the apoptotic neutrophil can regulate the inflammatory response and stimulate macrophages to form a pro-resolution phenotype capable of releasing pro-resolution mediators and cytokines (Porcheray et al., 2005, Benoit et al., 2008). Furthermore, models of inflammatory disease have demonstrated an anti-inflammatory capacity of the apoptotic neutrophil itself (Serhan et al., 2008). All of the above act to halt the inflammatory response, preventing inappropriate activation and exacerbation/progression of inflammation.

This thesis set out to further understand the mechanism through which the CDKi R-roscovitine induces apoptosis in neutrophils, a phenomenon previously observed in our laboratory. To date CDKis such as R-roscovitine are in late stage clinical trials for use as novel anti-cancer treatments and have proved successful at inhibiting proliferation and inducing apoptosis in carcinogenic cells (Krystof and Uldrijan, 2010). Our laboratory has highlighted a potential novel use for CDKis in the treatment of neutrophil dominant inflammatory diseases, through targeted induction of neutrophil apoptosis. A niche in the market exists for such a treatment, as diseases such as COPD do not respond well to treatment with traditional therapies such as glucocorticoids. A possible reason behind this may be that whilst glucocorticoid treatment increases removal of the apoptotic cells via macrophage phagocytosis, it actually inhibits neutrophil apoptosis (as demonstrated in Chapter 3) (S. Saffar et al., 2011). This creates a two fold problem: firstly neutrophils persist at the site of inflammation primed for activation, which in turn draws more neutrophils to the affected area (all capable of releasing their histotoxic arsenal of proteases and reactive oxygen products); secondly when the abundant neutrophils do finally enter apoptosis their copious numbers can overwhelm their removal by the macrophages

enabling the apoptotic cells to pass into secondary necrosis. If this occurs, the neutrophil's cytotoxic contents are liberated into the extra-cellular milieu which can result in further damage to the surrounding tissue and exacerbation of the inflammatory process.

In order for a novel treatment to be successful, it is essential that it does not impact negatively on the surrounding cellular environment. Therefore the first results chapter (Chapter 3) was concerned with the effects of R-roscovitine on the activation state of the neutrophil and the interaction of the apoptotic neutrophil with the phagocytic macrophage. The main observations and subsequent conclusions drawn are as follows: R-roscovitine does indeed result in death of neutrophils via apoptosis in as little as 4 h. This is a significant decrease compared to their circulating longevity *in vivo* which ranges from 7-12 h, and their lifespan in the tissues, which can exceed 24 h. Another aim of this chapter was to establish the effects of key inflammatory mediators on neutrophil apoptosis. This provided us with base line results from which the effects of the addition of R-roscovitine could be examined. Once it had been firmly established that R-roscovitine caused targeted apoptosis in neutrophils, the effect of R-roscovitine on several pertinent markers of neutrophil activation was investigated, namely neutrophil shape change, CD11b upregulation and CD62L shedding. Comparisons were made to the apoptosis inducing agent gliotoxin, to see if any similarities were present. It was found that when R-roscovitine was administered alone or in conjunction with GM-CSF, LPS, TNF- $\alpha$  or LTB<sub>4</sub> the anticipated activation parameters were unaffected (unlike gliotoxin). This suggests that R-roscovitine is acting in a different way to gliotoxin which does not stimulate a priming response in neutrophils. It also alludes to the possibility of R-roscovitine being well tolerated by neutrophils *in vivo*, not causing neutrophils to be untimely primed and susceptible to activation, which could contribute to tissue damage and exacerbation of the inflammatory process. Furthermore, as CD11b and CD62L are important for the processes of neutrophil adhesion to the vascular endothelium, the above results also infer that R-roscovitine is unlikely to disrupt the process of neutrophil recruitment and migration to sites of inflammation by inflammatory mediators. If this were to be disrupted, it could potentially result in an increased propensity of infection. This is an important and



promising finding when considering R-roscovitine as a possible novel treatment for neutrophil driven inflammatory disorders.

The potential suitability of R-roscovitine as a novel anti-inflammatory agent is further supported by the finding that R-roscovitine does not affect the ability of macrophages to phagocytose apoptotic neutrophils, or the phenomenon of dexamethasone increased phagocytosis. Interestingly, it appears that R-roscovitine may increase phagocytosis of apoptotic cells (observed in both the R-roscovitine condition and when R-roscovitine was co-administered with dexamethasone) when compared to the relevant controls. A possible explanation for this observation is that R-roscovitine increases the number of apoptotic cells present compared to the control condition. Subsequently apoptotic neutrophils are more abundant in the R-roscovitine treated conditions therefore, the macrophages engulf greater numbers. This is an exciting observation given the importance of the phagocytosis of apoptotic cells in triggering resolution of inflammation.

Once the potential suitability for R-roscovitine as a possible novel anti-inflammatory agent had been established the next step was to investigate the mechanism through which it acts. Due to the complex and redundant nature of the inflammatory response this task proved lengthy and intricate. However, in the final results chapter (Chapter 4) the following conclusions were arrived at. The initial suspicion that R-roscovitine was not directly inhibiting NF- $\kappa$ B was investigated and its effects on the classical/canonical pathway were assessed by I $\kappa$ B $\alpha$  degradation and via the translocation of p65 to the nucleus. Both methods confirmed that R-roscovitine does not directly activate the NF- $\kappa$ B pathway, nor does it inhibit the ability of LPS or TNF- $\alpha$  to activate NF- $\kappa$ B. This is an important finding as it implies that the canonical pathway, the pathway implicated in the inflammatory response, is not directly augmented by R-roscovitine. Furthermore, it indicates that the apoptosis inducing effect of R-roscovitine in the inflammatory neutrophil is via a different pathway to that proposed by Dai *et al.* in leukaemic cells (Dai et al., 2003). However, this does not rule out the possible involvement of NF- $\kappa$ B further downstream in the pathway, for example at the point of NF- $\kappa$ B dependent transcription. Interestingly though it may suggest that R-roscovitine's main mode of action is not through inhibition of one of the survival proteins

under the regulation of NF- $\kappa$ B (for example A1, X-IAP, cIAP1 and cIAP2) and that any subsequent down regulation of these proteins is associated with apoptosis already being triggered within the cell. To try and identify which survival proteins R-roscovitine targeted in the neutrophil, extensive studies were carried out on the following two survival proteins: Mcl-1, chosen because it is not thought to be under direct regulation of NF- $\kappa$ B; and X-IAP which is proposed to be transcriptionally activated by NF- $\kappa$ B. The latter protein was chosen, as whilst our provisional investigations eluded the NF- $\kappa$ B pathway not being directly influenced by R-roscovitine, it is possible that R-roscovitine may be exerting some effects further down the NF- $\kappa$ B pathway which are influencing levels of NF- $\kappa$ B transcribed survival proteins. Interestingly, it was found that Mcl-1 levels in the neutrophil decreased rapidly (in as little as 2 h) upon culturing with R-roscovitine, which is an important observation as this precedes observable apoptosis. Therefore, this implies that loss of Mcl-1 is caused by exposure to R-roscovitine and maybe important in initiating apoptosis and that this decrease is not just an artefact of a cell undergoing active apoptosis. Furthermore, R-roscovitine was able to over-ride the ability of GM-CSF to maintain Mcl-1 levels and in addition it appears that R-roscovitine can also down-regulate Mcl-1 in the presence of TNF- $\alpha$  and LPS. The ability of R-roscovitine to over-come powerful survival stimuli such as these could prove invaluable in its proposed application as a novel treatment for neutrophil dominant inflammatory disorders as it will enable R-roscovitine to induce neutrophil apoptosis even in severely inflamed areas where levels of survival factors, such as the ones above, are elevated. As it has been previously reported that protein levels of Mcl-1 can be regulated by the proteasome (Derouet et al., 2004) as well as by caspases (Herrant et al., 2004), provisional experiments were conducted into the mechanism through which R-roscovitine decreased levels of Mcl-1. Results imply that R-roscovitine induces caspase dependent reduction of Mcl-1. This is supported by the appearance of cleaved caspase 3 in R-roscovitine treated conditions. The involvement of X-IAP in R-roscovitine induced neutrophil apoptosis is less clear cut. Immunoblotting for X-IAP did suggest that X-IAP could potentially be reduced in the presence of R-roscovitine. It is possible that R-roscovitine may not be acting directly via X-IAP and that reductions observed are as a result of the apoptotic process already being initiated in the neutrophil. Results are further complicated by the fact that X-IAP is involved in the final stages of apoptosis and where

multiple pathways converge. To try and shed more light on the possible involvement of X-IAP in R-roscovitine induced apoptosis, HIV-tat transduction of an X-IAP-tat construct was attempted. Preliminary experiments revealed, as would be expected, that the introduction of X-IAP by the use of the tat construct significantly reduced neutrophil apoptosis at 18 h. In addition the X-IAP-tat construct appeared to partially reverse the induction of apoptosis. However, further investigations revealed contamination of the tat construct with LPS, a result of its bacterial vector production. As LPS is known to increase longevity of neutrophils the results obtained were confounded. Therefore, whilst it is possible that some of the longevity conferred on the neutrophils in the R-roscovitine + X-IAP-tat could be due to the increase in functional X-IAP protein, the results can not be fully contributed to this. In conclusion the results from Chapter 4 indicate that R-roscovitine is initiating caspase dependent apoptosis likely caused by a reduction in Mcl-1 resulting in subsequent but not causal reduction in X-IAP. Furthermore, whilst NF- $\kappa$ B may be implicated further down stream, the canonical pathway does not appear to be affected by R-roscovitine.

In summary, the work performed for this thesis supports the possible use of R-roscovitine as a novel treatment for neutrophil dominant inflammatory disorders. It indicates R-roscovitine would be well tolerated *in vivo* as an agent which effectively targets neutrophil apoptosis whilst not perturbing anticipated neutrophil activation parameters induced by key inflammatory mediators. Importantly R-roscovitine appears to have no detrimental effects on macrophage phagocytosis of apoptotic neutrophils, enabling effective removal of the apoptotic cells preventing them from entering secondary necrosis and further exacerbating the inflammatory response. In addition, this thesis begins to shed some light on the potential mechanism through which R-roscovitine induces apoptosis in neutrophils. It puts forward the idea that direct inhibition of NF- $\kappa$ B is not responsible for R-roscovitine driven apoptosis and adds to the wealth of literature indicating the importance of the survival protein Mcl-1 in neutrophils, implicating an essential role for the protein in R-roscovitine induced apoptosis. Excitingly these results hint at a possible novel two pronged approach to treatment of neutrophil dominant inflammatory diseases such as COPD, which as yet do not have a truly effective course of treatment. Administration of a CDK inhibitor such as R-

roscovitine would target induction of neutrophil apoptosis whilst subsequent treatment with a steroid, such as dexamethasone, would increase macrophage clearance of the apoptotic cells, kick starting the process of resolution.

## **5.2 Limitations**

There are several limitations associated with experimentation on primary neutrophils. One of the most regularly encountered, and the one which proves particularly troublesome when immunoblotting, is the abundance of proteases contained within neutrophils. Whilst these are undoubtedly necessary for the neutrophils vital immuno-defensive function they are exceedingly difficult to fully inhibit when producing lysates, even when a broad spectrum of proteases are employed. As one focus of this thesis was anti-apoptotic proteins, this was of particular concern as survival proteins such as Mcl-1 have a very short half life and are rapidly turned over, therefore extremely vulnerable to degradation by the proteases. Every possible step was taken to ensure the proteases were fully inhibited. However, this may help explain, at least in part, the lack of conclusive results when immunoblotting for X-IAP.

The human promyelocytic leukemia cell line (HL60) established in 1977 resembles promyelocytes and can be stimulated to differentiate into granulocyte type cells by compounds such as DMSO, retinoic acid and actinomycin D (Birnie, 1988). HL60 cells have been widely used as an alternative to primary neutrophils in numerous previous studies as they possess benefits such as being a homogenous pool therefore avoiding donor variations and, furthermore, inhibition of proteases may prove easier. However, our laboratory feels that they are not an ideal alternative for studying the role of the neutrophil in inflammation. This is because they are derived from a cancerous cell line and therefore it is highly likely that some of the cellular pathways/genes expression profiles will differ from wild type cells, especially those involved in apoptosis as cancer cells are immortal. Subsequently extrapolation of results to normally functioning cells would be tentative; this view is supported by Itoh K et al who, in 1998, demonstrated differential expression of genes between HL60 cells and granulocytes (Itoh et al., 1998).

### 5.3 Future Directions

Since the completion of the work reported in this thesis, our laboratory has confirmed and further investigated the effects and mechanisms of action of R-roscovitine. Data has been produced supporting the proposition in this thesis that R-roscovitine can overcome the presence of numerous survival factors, not just GM-CSF, to induce reduction of Mcl-1 protein. These include TNF- $\alpha$ , LPS and dbcAMP. Interestingly, R-roscovitine induced reduction of the Mcl-1 protein in the presence of survival factors is more marked than when neutrophils are incubated with R-roscovitine alone (Leitch et al., 2010). Furthermore, R-roscovitine has been found to have only a small effect on ERK phosphorylation in neutrophils (Leitch et al., 2010). This is despite reports that R-roscovitine can inhibit ERK (Bach et al., 2005). Our laboratory believes that R-roscovitine induces apoptosis at a downstream location within the neutrophil with suitable strength to overcome the increased survival associated with events upstream, such as activation of survival pathways by inflammatory mediators. The observation in this thesis that the reduction of Mcl-1 protein levels is an important step in the induction of neutrophil apoptosis by R-roscovitine is in keeping with this hypothesis. A future direction for this thesis (as noted below) was to investigate the effect of R-roscovitine on Mcl-1 transcription and this has since been progressed by our laboratory. It has been found that R-roscovitine reduced Mcl-1 mRNA and this decrease can be seen in as little as 2 h post treatment. Therefore, it can be concluded that R-roscovitine is influencing transcription of the Mcl-1 gene and that the decrease in the Mcl-1 protein observed in this thesis is a consequence of this. In addition the potential use of R-roscovitine as an anti-inflammatory agent that aides resolution of neutrophilic inflammation has been confirmed in our laboratory through the use of murine models of arthritis, lung fibrosis and pleurisy, in which therapeutic benefits associated with an increase of neutrophil apoptosis were observed (Rossi et al., 2006).

Furthermore, others have shown that neutrophil induced apoptosis by CDKis can be beneficial in multiple disease models. For example, *in vivo* work on murine models of pneumococcal infection has found that induction of neutrophil apoptosis by R-roscovitine in combination with antibiotic treatment improves resolution of inflammation and recovery

from meningitis (Koedel et al., 2009). This highlights the potential benefits of a double pronged approach to the treatment of inflammatory diseases in which additional agents such as steroids or antibiotics could be used to tailor the actions of CDKis to the specific needs of the disease. In addition, R-roscovitine has also been found to induce apoptosis in neutrophils from cystic fibrosis patients, a condition characterised by neutrophil dominant inflammation driven by powerful CF associated mediators, portraying that R-roscovitine can overcome neutrophil induced survival by a plethora of inflammatory mediators, not just those reported in this thesis (Moriceau et al., 2010).

It is not just our knowledge regarding the anti-inflammatory effects of CDKis that has increased over the past few years; exciting advances have also been made in the techniques available to study inflammatory responses. One of the most prevalent advances made is the zebrafish model of neutrophilic inflammation which allows the inflammatory response to be followed from start to finish. Renshaw *et al.* devised a model in transgenic zebrafish using the myeloperoxidase promoter to produce GFP, therefore enabling neutrophils to be tracked. This model has been successfully used to portray R-roscovitine induced neutrophil apoptosis and subsequent resolution of inflammation (Loynes et al., 2010).

We are also discovering that the anti-inflammatory benefits of R-roscovitine are not limited to the induction of neutrophil apoptosis. A current lead being pursued within the laboratory is the observed decrease in production of cytokines induced by CDKis in human macrophages (data unpublished); this falls in line with observations made by various other groups who have reported the anti-inflammatory benefits of CDKis in cells such as the macrophages. For example Lloberas and Celada have shown that p21 can act as a negative regulator of macrophage activation (Lloberas and Celada, 2009). Another area of research in which CDKis have been implicated in the inflammatory response in ways other than an increase neutrophil apoptosis is rheumatoid arthritis. Sekine *et al.* found that flavopiridol and a specific CDK4,6 inhibitor could alleviate rheumatoid arthritis in various models by a lymphocyte independent mechanism which reduces fibroblast growth and proliferation (Sekine et al., 2008). In addition a notable paper has been produced by Schmerwitz *et al.* who found that flavopiridol (a non-specific CDKi) targets endothelial cells inhibiting

expression of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin. This has important anti-inflammatory implications as the above factors are necessary for the successful migration of neutrophils to sites of inflammation. Furthermore, they found NF- $\kappa$ B dependent transcription was blocked. The latter finding is of particular interest as they found p65 translocation, degradation and phosphorylation of I $\kappa$ B $\alpha$ , and the ability of NF- $\kappa$ B to bind to DNA to be unaffected (Schmerwitz et al., 2011). This is significant as one of the observations in this thesis was that R-roscovitine did not affect degradation of I $\kappa$ B $\alpha$  or the translocation of p65 to the nucleus, therefore did not appear to be having a direct effect on NF- $\kappa$ B signalling. However, this could indicate that NF- $\kappa$ B dependent transcription could still be being prevented by R-roscovitine. They elucidated through use of a kinome array and kinase activity panel, followed by subsequent inhibition of the identified kinases, that flavopiridol was causing the above effects through inhibition of CDK9 (Schmerwitz et al., 2011). Interestingly CDK9 is also present in neutrophils and within the specificity of R-roscovitine.

In summary, all of the above findings highlight the importance of further research into the anti-inflammatory capabilities of CDKis such as R-roscovitine.

If time constraints were not present the experiments presented in this thesis would be progressed in the following ways:

- Real Time (rt)PCR would be used to investigate expression of survival proteins such as Mcl-1 and X-IAP at the level of translation in neutrophils treated with R-roscovitine.

In this thesis the effects of R-roscovitine on Mcl-1 and X-IAP expression has been examined at a translational level. In order to establish if the decrease in Mcl-1 protein observed upon neutrophil treatment with R-roscovitine is a consequence of R-roscovitine reducing the amount of Mcl-1 mRNA transcribed (rt)PCR would be used thereby allowing the transcribed levels of Mcl-1 mRNA to be quantified.

It is highly likely that a reduction of Mcl-1 at the translational level would be evident as CDK 7 and CDK 9 are required for phosphorylation of the carboxyl-terminal domain of RNA Pol II. This is important for the binding of transcription factors and for the modification of mRNA (such as splicing and capping), thus perturbation of these essential phosphorylations would lead to inhibition of RNA synthesis and thus a decrease in cellular levels of the protein which it is translated into. This would have particular impact on proteins which are subject to rapid turn over, such as Mcl-1 which has a notably short half life for both its mRNA and protein. Given that CDKs 1, 2, 5, 7 and 9 fall within the inhibitory range of R-roscovitine it is probable that in cells exposed to this trisubstituted purine analogue transcription by RNA Pol II will be disrupted (MacCallum et al., 2005). As mentioned above this has since been confirmed by our laboratory; Leitch *et al.* demonstrated in 2010 that Mcl-1 mRNA is reduced by R-roscovitine in just 2 h, therefore supporting our hypothesis that a reduction of Mcl-1 is evident before the onset of apoptosis and thus is a causative factor in its initiation (Leitch et al., 2010).

- Further investigation into the specific roles of CDK7 and CDK 9 in human neutrophils.

The previous implication that R-roscovitine could be inhibiting CDK7 and CDK9 phosphorylation of RNA pol II would be further investigated by Western Blotting and Confocal Microscopy to see if this could be a causative factor in the reduction of Mcl-1 observed in primary human neutrophils treated with R-roscovitine. In light of the work produced by Schmerwitz *et al.* a kinome assay and kinase activity screen of the neutrophil to produce the protein kinases present in the neutrophils genome would also be a beneficial exercise. This would allow, through subsequent inhibition of the kinases in the kinome, identification of the kinase target of R-roscovitine in human neutrophils.

- Investigate further if NF- $\kappa$ B could be implicated in the scope of R-roscovitine's action.



This would be performed due to the discovery by Schmerwitz *et al* that Flavopiridol was inhibiting NF- $\kappa$ B dependent transcription in endothelial cells in the absence of I $\kappa$ B $\alpha$  degradation and whilst mobilisation of p65 was still evident. This could potentially be due to inhibition of CDK9 and again a kinome screen/kinase inhibition approach would help determine if this was the case.

- Experiments would be repeated in eosinophils to see if R-roscovitine exhibits a similar profile of effects to those it elicits in the neutrophil.

Exploration of the effects of R-roscovitine on eosinophil apoptosis would be used to try and establish similarities and differences in the regulation of the two granulocytes which may ultimately have the potential to be exploited for therapeutic gain. Our laboratory has recently demonstrated that R-roscovitine does indeed induce eosinophil apoptosis in a concentration and time dependent manner and that, as with neutrophils, there is a reduction in Mcl-1 levels prior to the onset of apoptosis (Duffin *et al.*, 2009). This has promising implications for the potential use of R-roscovitine as a novel treatment to aid resolution of eosinophil driven inflammation.

- Treatment of neutrophils with the R-roscovitine metabolite in which the hydroxyl-group at C2 has been converted to carboxylic acid.

Vita M. *et al* in 2005 demonstrated that rats administered intravenous R-roscovitine (14.12 $\mu$ M) metabolise and eliminate it from the plasma and tissue in less than 30 min. Three metabolites were observed, of these three the main one found in plasma was termed M1 where the hydroxyl-group at C2 has been converted to carboxylic acid. The other two were mainly observed in the liver, kidneys and several other organs. It is possible neutrophils may be converting R-roscovitine into a metabolite such as M1 and that this could potentially be triggering apoptosis. High performance liquid chromatography could be used to identify any metabolites produced within neutrophils (Vita *et al.*, 2005).

- Use of a protein transduction domain to introduce survival proteins such as Mcl-1 and X-IAP into neutrophils and observe effects of R-roscovitine.

As R-roscovitine could be inhibiting the transactivation function of HIV-tat proteins which requires CDK9 and T1 (Canduri et al., 2008) a different method of protein transduction should be attempted, ideally one which can be produced in a non bacterial vector to prevent LPS contamination. A potential alternative method of transduction is by lentivirus, Dick *et al.* have successfully and reliably transfected primary human neutrophils with a range of constructs *via* lenivirus, including GFP-fusion proteins (Dick et al., 2009). This would enable us to introduce X-IAP-GFP proteins into neutrophils and allow us to locate and quantify the tagged protein.

- Investigate the presence of Mcl-1<sub>s</sub> and its pro-apoptotic role in neutrophil apoptosis, and if this is augmented by the presence of R-roscovitine.

This would be performed to help us elicit if it is in fact the pro-apoptotic Mcl-1<sub>s</sub> splice variant (Bingle et al., 2000) that is involved in the rapid induction of apoptosis by R-roscovitine in neutrophils. It would also enable us to see if there is a preferential Mcl-1 target for R-roscovitine.

## 5.4 Concluding Remarks

In conclusion this thesis has confirmed the hypothesis that CDKis such as R-roscovitine can efficaciously induce apoptosis in human neutrophils through regulation of critical survival proteins, and has identified Mcl-1 as a key player in this process. Furthermore it has demonstrated promising results for the tolerability of R-roscovitine as a potential novel treatment for human inflammatory conditions arising from neutrophilic inflammation. This supports the long-term aim of this thesis, and our laboratory, to take R-roscovitine to clinical trial as a potential new therapy for neutrophil dominant inflammatory disorders, and excitingly this is now being progressed within our institute.

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**Publications arising from this thesis**

# Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis

Adriano G Rossi<sup>1</sup>, Deborah A Sawatzky<sup>1,3</sup>, Annemieke Walker<sup>1,3</sup>, Carol Ward<sup>1</sup>, Tara A Sheldrake<sup>1</sup>, Nicola A Riley<sup>1</sup>, Alison Caldicott<sup>1</sup>, Magdalena Martinez-Losa<sup>1</sup>, Trevor R Walker<sup>1</sup>, Rodger Duffin<sup>1</sup>, Mohini Gray<sup>1</sup>, Elvira Crescenzi<sup>2</sup>, Morag C Martin<sup>1</sup>, Hugh J Brady<sup>2</sup>, John S Savill<sup>1</sup>, Ian Dransfield<sup>1</sup> & Christopher Haslett<sup>1</sup>

Apoptosis is essential for clearance of potentially injurious inflammatory cells and subsequent efficient resolution of inflammation. Here we report that human neutrophils contain functionally active cyclin-dependent kinases (CDKs), and that structurally diverse CDK inhibitors induce caspase-dependent apoptosis and override powerful anti-apoptosis signals from survival factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF). We show that the CDK inhibitor *R*-roscovitine (Seliciclib or CYC202) markedly enhances resolution of established neutrophil-dependent inflammation in carrageenan-elicited acute pleurisy, bleomycin-induced lung injury, and passively induced arthritis in mice. In the pleurisy model, the caspase inhibitor zVAD-fmk prevents *R*-roscovitine-enhanced resolution of inflammation, indicating that this CDK inhibitor augments inflammatory cell apoptosis. We also provide evidence that *R*-roscovitine promotes apoptosis by reducing concentrations of the anti-apoptotic protein Mcl-1. Thus, CDK inhibitors enhance the resolution of established inflammation by promoting apoptosis of inflammatory cells, thereby demonstrating a hitherto unrecognized potential for the treatment of inflammatory disorders.

Neutrophils have a central role in innate immunity and are rapidly recruited to sites of infection and injury; however, their many defense mechanisms that destroy and digest invading microorganisms are potentially deleterious to tissues<sup>1</sup>. Thus, it is vital that, once their physiological function has been achieved, these inflammatory cells and their potentially histotoxic contents are cleared rapidly. During spontaneous resolution of inflammation, neutrophils undergo apoptosis: a pre-programmed and highly regulated cell death process that results in shutdown of secretory capacity and allows recognition and removal by macrophages<sup>2,3</sup>. Neutrophil survival and apoptosis are profoundly influenced by the inflammatory milieu: inflammatory mediators such as GM-CSF or lipopolysaccharide (LPS), environmental conditions such as hypoxia, and the presence of pro-apoptotic stimuli such as tumor necrosis factor- $\alpha$  or Fas ligand markedly alter neutrophil longevity<sup>4,5</sup>.

Neutrophil apoptosis is controlled by a complex network of signaling pathways that regulate both the turnover of key molecules, including the anti-apoptotic protein myeloid cell leukemia 1 (Mcl-1) and the pro-apoptotic Bcl-2 family member Bax, and activation of the caspase family of proteases<sup>5</sup>. Once apoptosis has been engaged, the neutrophil secretory activity is shutdown; the cells remain intact and are phagocytosed by macrophages using recognition mechanisms that

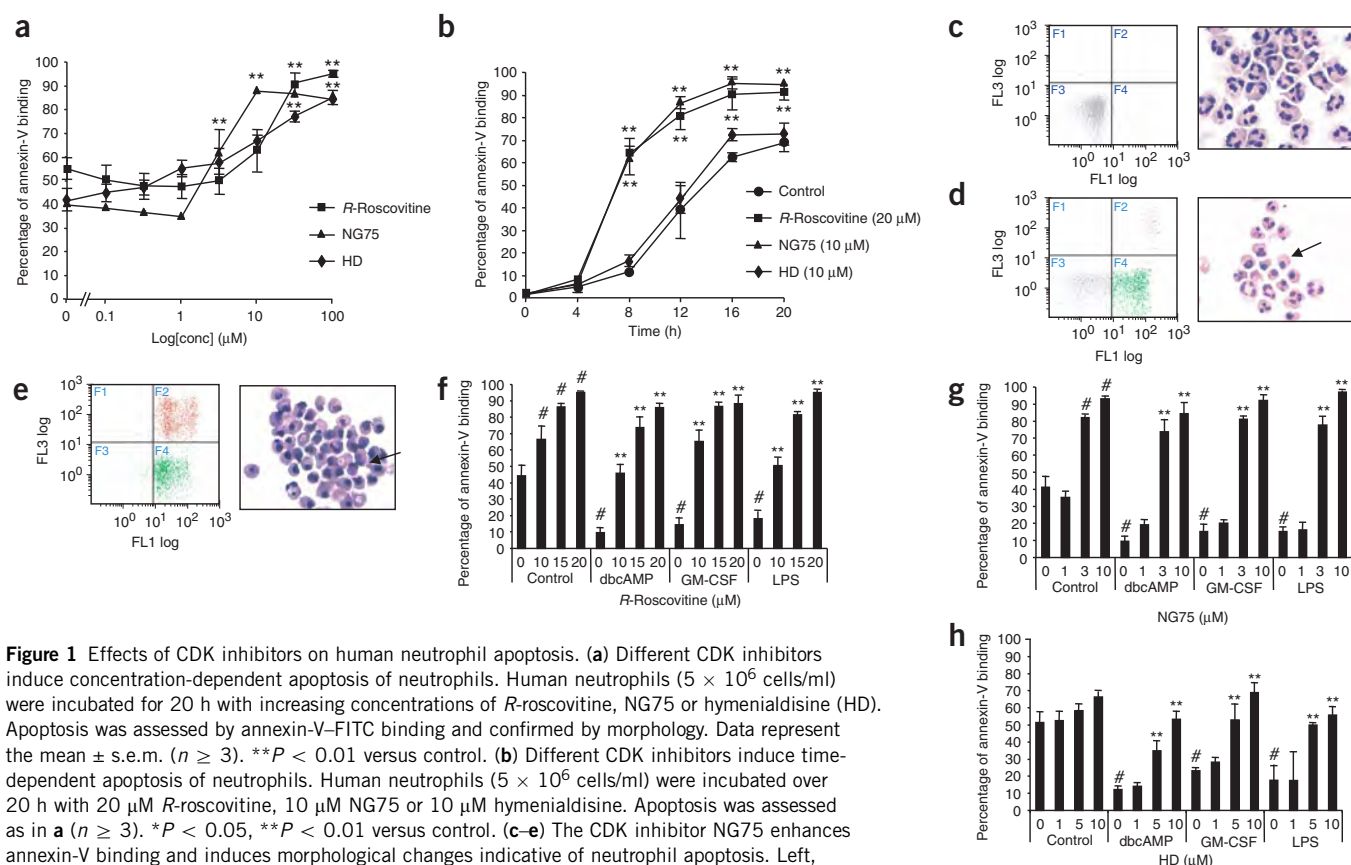
fail to elicit a pro-inflammatory response<sup>2,6</sup>. If macrophage phagocytosis or neutrophil apoptosis is impaired, however, chronic inflammation may ensue<sup>4,5,7</sup>. Consequently, the mechanisms involved in regulating inflammatory cell survival and apoptosis are the subject of considerable research endeavor.

Cell division of eukaryotic cells occurs in four phases (G1, S, G2, M) and in some circumstances, for example where growth factors are withdrawn or the cell is terminally differentiated, the cell will rest in G0 phase. The CDKs have been traditionally described as key regulators of the cell cycle, whereby different CDKs become activated during cell-cycle progression when complexed with their associated cyclin partners<sup>8</sup>. For this reason, targeting CDKs by specific inhibitors may prevent or limit tumor progression. Indeed, CDK inhibitors are under clinical trial for esophageal, lung, prostate and non-small-cell lung cancers<sup>9</sup>. In contrast, inhibition of CDKs attenuates apoptosis of terminally differentiated neurons<sup>10</sup>. The precise mechanisms that determine the effects of CDK inhibitors on apoptosis remain unclear, although these inhibitors downregulate Mcl-1 (refs. 11,12), a key protein regulating apoptosis<sup>13</sup> including neutrophil apoptosis<sup>14</sup>. Neutrophils are terminally differentiated cells and therefore CDK inhibitors such as *R*-roscovitine would be predicted either to have no effect or, as in neurons, to inhibit apoptosis<sup>10</sup>.

<sup>1</sup>MRC Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK.

<sup>2</sup>Molecular Haematology and Cancer Biology Unit, Institute of Child Health, Great Ormond Street Hospital for Children NHS Trust, University College London, 30 Guilford Street, London WC1N 1EH, UK. <sup>3</sup>These authors contributed equally to this article. Correspondence should be addressed to A.G.R. (a.g.rossi@ed.ac.uk).

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**Figure 1** Effects of CDK inhibitors on human neutrophil apoptosis. **(a)** Different CDK inhibitors induce concentration-dependent apoptosis of neutrophils. Human neutrophils ( $5 \times 10^6$  cells/ml) were incubated for 20 h with increasing concentrations of *R*-roscovitrine, NG75 or hymenialdisine (HD). Apoptosis was assessed by annexin-V-FITC binding and confirmed by morphology. Data represent the mean  $\pm$  s.e.m. ( $n \geq 3$ ).  $**P < 0.01$  versus control. **(b)** Different CDK inhibitors induce time-dependent apoptosis of neutrophils. Human neutrophils ( $5 \times 10^6$  cells/ml) were incubated over 20 h with 20  $\mu\text{M}$  *R*-roscovitrine, 10  $\mu\text{M}$  NG75 or 10  $\mu\text{M}$  hymenialdisine. Apoptosis was assessed as in **a** ( $n \geq 3$ ).  $*P < 0.05$ ,  $**P < 0.01$  versus control. **(c–e)** The CDK inhibitor NG75 enhances annexin-V binding and induces morphological changes indicative of neutrophil apoptosis. Left, flow cytometry dot plots of NG75-treated neutrophils, taken at 0 h (**c**), 8 h (**d**) and 20 h (**e**). Non-apoptotic cells are shown in gray, annexin-V-FITC<sup>+</sup> cells in green, and annexin-V-FITC<sup>+</sup>/PI<sup>+</sup> cells in red. Right, corresponding micrographs. In **c**, typical neutrophil morphology of multi-lobed nuclei is seen. In **d**, arrow indicates typically apoptotic neutrophil morphology, showing condensed nuclei. In **e**, arrow indicates apoptotic neutrophil without nuclear staining and the cell membrane appears 'leaky'. This type of morphology may account for the annexin-V-FITC<sup>+</sup>/PI<sup>+</sup> cells seen by flow cytometry at 20 h. **(f–h)** CDK inhibitors reverse dbcAMP-, GM-CSF- and LPS-mediated survival of neutrophils. Human neutrophils ( $5 \times 10^6$  cells/ml) were pre-incubated with 0.2 mM dbcAMP, 50 U/ml of GM-CSF or 1  $\mu\text{g}/\text{ml}$  of LPS as indicated for 30 min. Appropriate concentrations of *R*-roscovitrine (**f**), NG75 (**g**) or hymenialdisine (**h**) were then added and the cells were incubated for a further 20 h. Apoptosis was assessed as in **a** ( $n \geq 3$ ).  $\#P < 0.05$  versus control.  $*P < 0.05$ ,  $**P < 0.01$  versus control of relevant survival agent.

Here we have investigated whether CDK inhibitors can influence neutrophil apoptosis *in vitro* and consequently the resolution of neutrophilic inflammation *in vivo*. We show that human neutrophils express functional CDKs and that different CDK inhibitors directly induce caspase-dependent neutrophil apoptosis and inhibit cell survival induced by several biologically important powerful anti-apoptotic agents. We also show that the CDK inhibitor *R*-roscovitrine downregulates Mcl-1 expression induced by survival factors in neutrophils. In addition, we demonstrate *in vivo* that *R*-roscovitrine markedly enhances resolution of inflammation in mouse models of carrageenan-induced acute pleurisy, bleomycin-induced lung inflammation and passively induced arthritis. The *R*-roscovitrine-enhanced resolution of established pleurisy is driven by a caspase-mediated pro-apoptotic effect. These findings suggest that CDK inhibitors may provide a therapeutic strategy to promote resolution of inflammatory diseases.

## RESULTS

### CDK inhibitors induce human neutrophil apoptosis

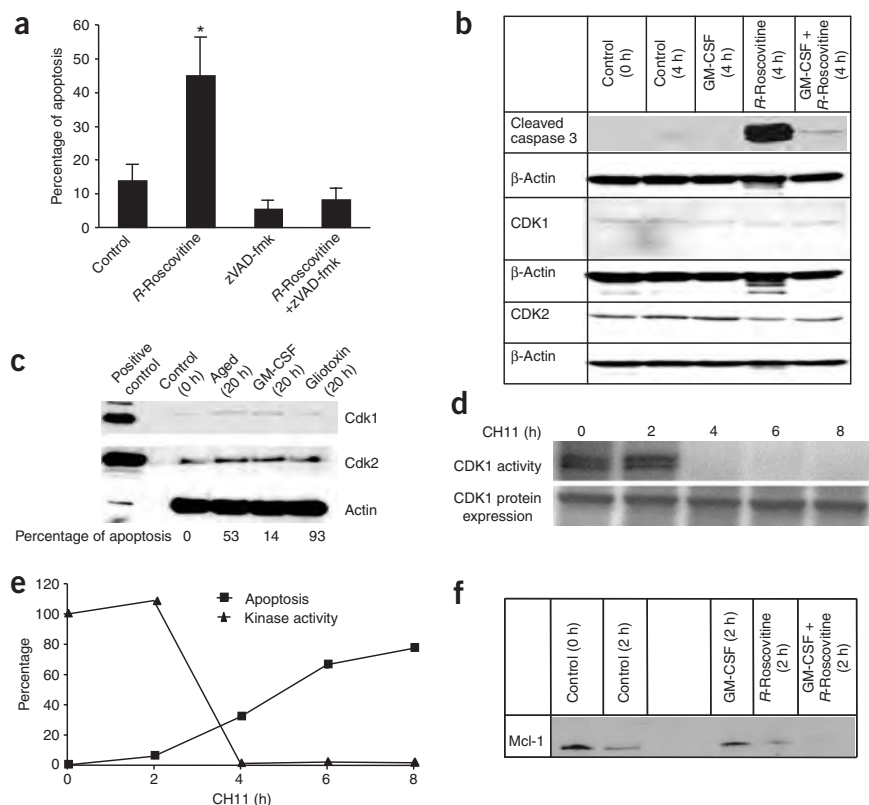
To investigate whether CDK inhibitors can affect apoptosis directly, human neutrophils were incubated over a 20-h period with the structurally diverse CDK inhibitors *R*-roscovitrine<sup>15–17</sup>, NG75 (refs.

18,19) and hymenialdisine<sup>20,21</sup> at a range of concentrations similar to those known to inhibit CDKs specifically<sup>15–21</sup>. The CDK inhibitors markedly increased neutrophil apoptosis in a concentration-dependent (Fig. 1a) and time-dependent (Fig. 1b) manner. After 8 h of NG75 treatment, a marked increase in annexin-V-positive cells was observed (Fig. 1c–e); after prolonged treatment (20 h), an increased number of annexin-V- and propidium iodide (PI)-positive cells (indicating secondary necrosis) were present and these cells could be identified morphologically by their nuclear loss and ruffled plasma membrane (Fig. 1e). Similar profiles were seen with *R*-roscovitrine and hymenialdisine treatment, and all annexin-V binding data was confirmed by morphological assessment of apoptosis (data not shown).

In a time-course study, *R*-roscovitrine (20  $\mu\text{M}$ ) and NG75 (10  $\mu\text{M}$ ) increased the rate of apoptosis after 8 h, whereas 10  $\mu\text{M}$  hymenialdisine did not increase apoptosis above the control rate (Fig. 1b). We deliberately used hymenialdisine at 10  $\mu\text{M}$ , a concentration that did not affect apoptosis *per se*, because subsequently we wanted to test the effect of the CDK inhibitor on delayed apoptosis induced by survival factors (see below). Systematic study of its cellular targets has shown that *R*-roscovitrine has high specificity for CDK1, CDK2 and CDK5, but not for other kinases including CDK4 and CDK6 (refs. 15–17); in addition, both NG75 (refs. 18,19) and



**Figure 2** CDK protein and activity in neutrophils and mechanisms governing the pro-apoptotic effect of CDK inhibitors. **(a)** The caspase inhibitor zVAD-fmk inhibits *R*-roscovitine-induced apoptosis of neutrophils. Human neutrophils ( $5 \times 10^6$  cells/ml) were incubated for 6 h with *R*-roscovitine (20  $\mu$ M) with or without zVAD-fmk (100  $\mu$ M). Apoptosis was assessed as in **Fig. 1a** ( $n = 4$ ). \* $P < 0.05$  versus control. **(b)** CDK1 and CDK2 proteins are expressed in neutrophils and *R*-roscovitine induces caspase cleavage in neutrophils. Human neutrophils ( $5 \times 10^6$  cells/ml) were lysed after isolation (0 h) or after treatment (4 h) with buffer, GM-CSF (50 ng/ml), *R*-roscovitine (20  $\mu$ M), or GM-CSF (50 ng/ml) plus *R*-roscovitine (20  $\mu$ M). Proteins were subjected to western blotting with antibodies to caspase 3, CDK1, CDK2 or  $\beta$ -actin. Blots are representative of at least three separate experiments. **(c)** CDK1 and CDK2 protein expression does not change during neutrophil apoptosis. Human neutrophils ( $5 \times 10^6$  cells/ml) were lysed after isolation (0 h) or after treatment (20 h) with buffer, GM-CSF (50 U/ml) or gliotoxin (0.1  $\mu$ g/ml). Proteins were subjected to western blotting with antibodies to CDK1 or CDK2. Blots are representative of at least three separate experiments. **(d,e)** CDK1 activity is present in neutrophils and decreases after apoptosis induced by the activating antibody to Fas (CH11). Neutrophils ( $5 \times 10^6$  cells/ml) were treated with CH11 (500 ng/ml) for the indicated times. Subsequently,  $10^7$  cells were lysed for each sample, the lysate was incubated with antibody to CDK1, and CDK1 activity was measured by  $^{32}$ P transfer to histone H1 **(d,e)**. Apoptosis (annexin-V $^+$ /PI $^+$ ) **(e)** and CDK1 activity **(d,e)** were measured every 2 h over 8 h. CDK1 activity was detected at time 0 and at 2 h, but not at later time points as apoptosis increased **(d,e)**. Again, no difference in CDK1 expression was detected over time **(d)**. Results are representative of at least three separate experiments. **(f)** *R*-Roscovitine reduces survival factor-induced Mcl-1 protein expression in neutrophils. Human neutrophils ( $5 \times 10^6$  cells/ml) were treated with buffer, GM-CSF (50 ng/ml), *R*-roscovitine (20  $\mu$ M), or GM-CSF (50 ng/ml) plus *R*-roscovitine (20  $\mu$ M) for 2 h. After cell lysis, proteins were subjected to western blotting with antibody to Mcl-1. Blots are representative of at least three separate experiments.



hymenialdisine<sup>20,21</sup> have high specificity for the same CDKs. The crystal structure of human CDK2 complexed with *R*-roscovitine has been described, together with evidence showing that the *R* stereoisomer of roscovitine is slightly more potent at inhibiting purified CDK1/cyclin B activity than is the *S* stereoisomer (half-maximal inhibitory concentrations of 0.45  $\mu$ M and 0.95  $\mu$ M, respectively<sup>15</sup>). Our own studies with the stereoisomers used at 20  $\mu$ M showed that apoptosis after 6 h was  $12.4 \pm 0.4\%$  for the control,  $12.8 \pm 1.3\%$  for the dimethyl sulfoxide (DMSO; 0.04%) control,  $75.9 \pm 3.5\%$  for *R*-roscovitine and  $75.6 \pm 3.3\%$  for *S*-roscovitine (mean  $\pm$  s.e.m.;  $n = 4$  separate donors, done in triplicate).

#### CDK inhibitors overcome survival factor delay of apoptosis

Because these CDK inhibitors accelerated apoptosis, we investigated whether they reversed the effects of agents that delay neutrophil apoptosis through different signaling pathways<sup>5</sup>. For example, dbcAMP penetrates the cell membrane to mimic endogenous cAMP<sup>22</sup>; GM-CSF acts through the GM-CSF receptor to activate phosphatidylinositol 3-kinase (PI3K), Janus kinase 2 and the transcription factor STAT1 (ref. 5); and LPS binds to Toll-like receptor-4 to activate mitogen-activation kinase (MAPK), PI3K and NF- $\kappa$ B signaling to inhibit apoptosis<sup>5,23–26</sup>. In addition, LPS-induced delay of neutrophil apoptosis is also dependent on contaminating monocytes<sup>25,26</sup>. The CDK inhibitors overrode all of these survival signals in a concentration-dependent manner (**Fig. 1f–h**) without directly inducing apoptosis *per se* (see 10  $\mu$ M hymenialdisine data). Thus, overriding of survival

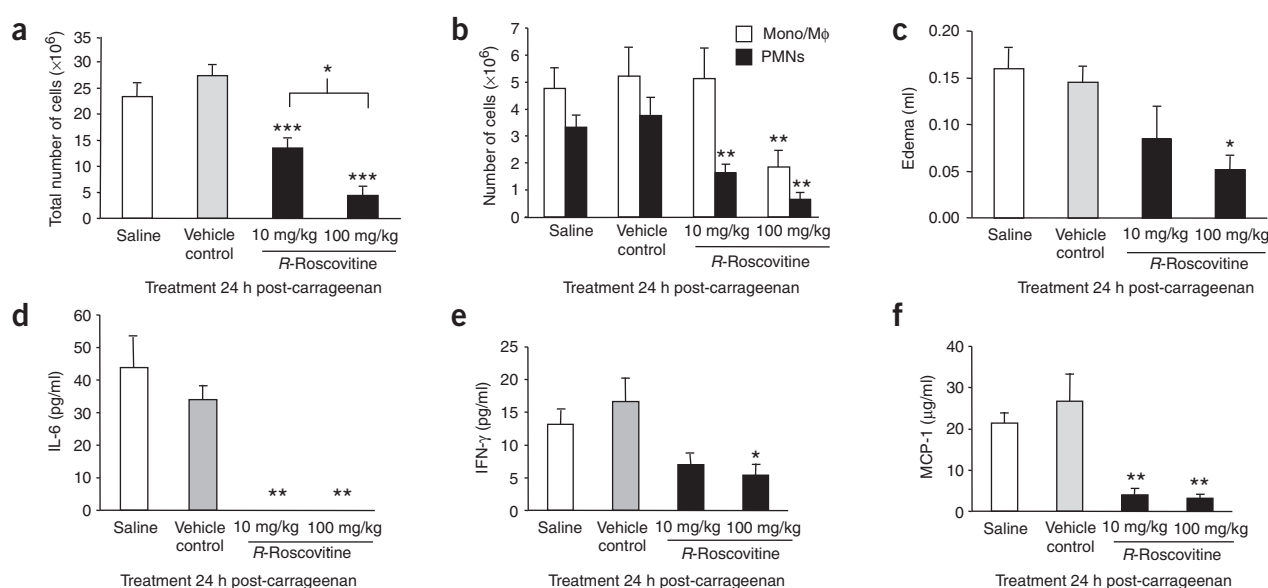
signals by CDK inhibitors occurs independently of, or is more sensitive to, direct effects of the inhibitors on apoptosis and takes place irrespective of the signaling pathways triggered in neutrophils.

#### CDK inhibitors induce caspase-dependent apoptosis

We found that CDK inhibitor-induced apoptosis was caspase dependent because pre-incubating neutrophils with the broad-range caspase inhibitor zVAD-fmk prevented *R*-roscovitine-induced apoptosis (**Fig. 2a**). *R*-Roscovitine treatment resulted in caspase-3 cleavage, directly verifying that this CDK inhibitor activates caspases in neutrophils (**Fig. 2b**). In addition, we found that *R*-roscovitine-induced caspase-3 cleavage was attenuated by the pro-survival factor GM-CSF (**Fig. 2b**), probably because of competing pro- and anti-apoptotic pathways. At the early time point of 4 h, the rate of basal apoptosis was low ( $< 5\%$ ; **Fig. 1b**) and consequently caspase-3 cleavage was minimal (**Fig. 2b**); however, caspase-3 cleavage was already very evident at 4 h when the neutrophils were treated with *R*-roscovitine alone. At 20 h, the pro-apoptotic effect of *R*-roscovitine was dominant over survival factor-mediated effects (**Fig. 1f**).

#### Human neutrophils express functionally active CDKs

Because neutrophils are terminally differentiated and do not undergo cell division, little is known about CDKs and their associated partners in these cells. CDK1 and CDK2 were found to be present in neutrophils (**Fig. 2b,c**) and there was no difference in CDK protein



**Figure 3** Effect of the CDK inhibitor *R*-roscovitine on resolution of carrageenan-induced pleurisy. (**a–c**) *R*-Roscovitine dose-dependently promotes resolution of inflammation *in vivo*. Twenty-four hours after intrapleural injection of 1% carrageenan, male C57/bl6 mice were treated with saline, DMSO, or 10 or 100 mg per kg body weight (mg/kg) of *R*-roscovitine (i.p.). Mice were killed 36 h after carrageenan injection and the total pleural inflammatory cell numbers (**a**), mononuclear (Mono/M $\phi$ ) and polymorphonuclear cell (PMNs) numbers (**b**) and exudate volumes (**c**) were measured. Data represent the mean  $\pm$  s.e.m. ( $n = 8$ –10 mice per group). \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus DMSO control. (**d–f**) *R*-Roscovitine reduces pro-inflammatory cytokines *in vivo*. Mice were treated as in **a–c**, and IL-6 (**d**), IFN- $\gamma$  (**e**) and MCP-1 (**f**) were measured in pleural exudates with a cytokine bead assay ( $n = 8$ –10 mice per group). \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus DMSO control. (**g**) *R*-Roscovitine promotes resolution of inflammation *in vivo* in a time-dependent manner. Twenty-four hours after intrapleural injection of 1% carrageenan, male C57/bl6 mice were treated with vehicle control (DMSO) or 100 mg per kg body weight of *R*-roscovitine (i.p.). Mice were killed 24 h, 48 h or 168 h after carrageenan injection and the total pleural inflammatory cell numbers were determined ( $n = 5$ –6 mice per group). \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus DMSO control.

quantities in freshly isolated neutrophils, aged neutrophils (4 h), or neutrophils treated with GM-CSF, *R*-roscovitine or both (4 h). Even when neutrophils were incubated for 20 h with buffer or GM-CSF, or when apoptosis was induced with gliotoxin<sup>23</sup>, the quantities of CDKs did not change (Fig. 2c). Thus, although CDKs seem to be important for inducing apoptosis, CDK1 and CDK2 are not targets for degradation during apoptosis.

In other cell systems, CDK1 and CDK2 have been shown to bind to various cyclin subtypes, whereas CDK5 does not seem to bind to cyclins but is activated by the non-cyclin p35 and p39 regulatory proteins. Because *R*-roscovitine can also inhibit CDK5 (ref. 16), we verified that CDK5 is expressed in human neutrophils (data not shown), in agreement with a previous study<sup>27</sup> that demonstrated the presence of CDK5 mRNA and protein. CDK1 (Fig. 2d,e), CDK2 (not shown) and CDK5 (ref. 27) activity has been shown in neutrophils. We found that CDK1 activity in neutrophils decreased rapidly during apoptosis induced by the activating Fas antibody CH11 (Fig. 2e). The CDK-binding partners and regulatory proteins in neutrophils remain to be identified.

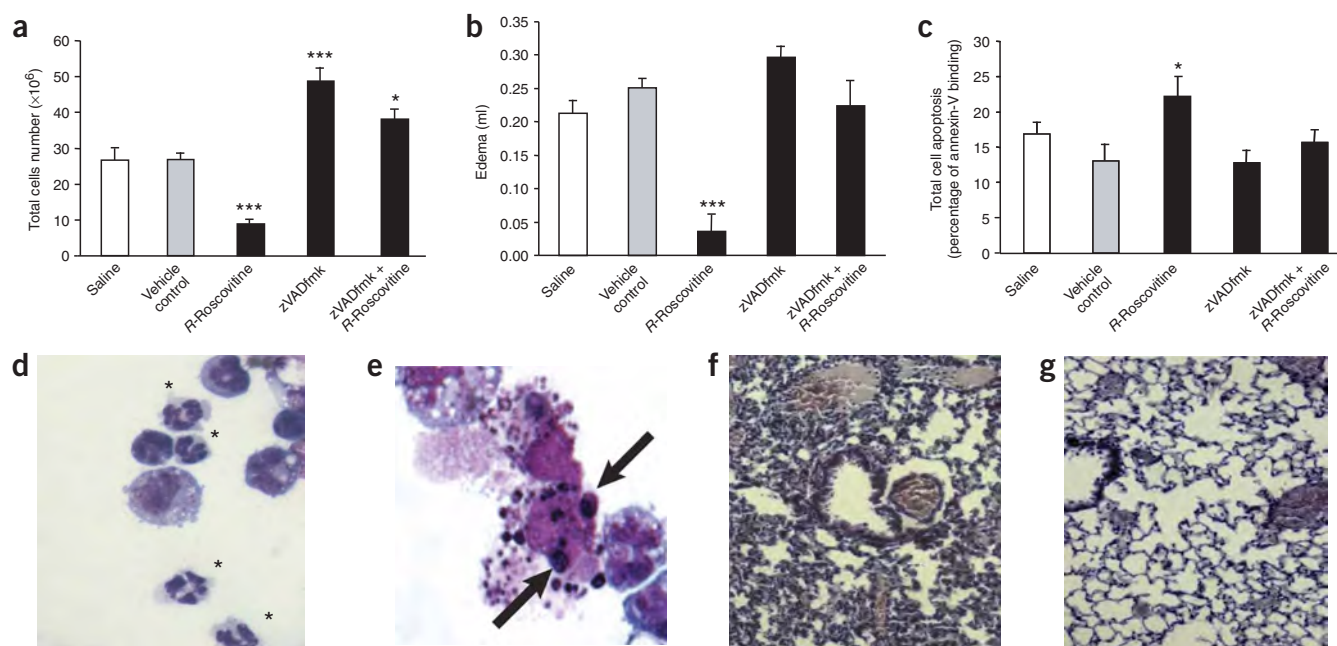
#### CDK inhibitors reduce neutrophil Mcl-1 expression

We further probed the effect of *R*-roscovitine on the expression of a key regulator of apoptosis, Mcl-1 (refs. 13,14), and found that Mcl-1

quantities in isolated cells fell rapidly within 2 h of culture, an effect that was prevented by GM-CSF treatment. *R*-Roscovitine inhibited GM-CSF-mediated upregulation of Mcl-1 (Fig. 2f).

#### Roscovitine-induced apoptosis enhances inflammatory resolution

Having shown *in vitro* that neutrophil apoptosis was markedly influenced by CDK inhibitors, we determined the effects of *R*-roscovitine on the resolution of neutrophil-dominant inflammation *in vivo*. We used a well-established acute resolving mouse model of carrageenan-induced pleural inflammation<sup>28–31</sup> to assess the effects of the CDK inhibitor on the kinetics of inflammatory cell recruitment and to investigate inflammatory mechanisms. *R*-Roscovitine accelerated the resolution of established inflammation when administered intraperitoneally (i.p.) 24 h after intrapleural injection of 1% carrageenan (Fig. 3). Thus, treatment with 10 mg per kg body weight of *R*-roscovitine (i.p.) inhibited the total inflammatory cell number by more than 50% as compared with vehicle control (Fig. 3a), and also reduced the number of monocytes/macrophages and neutrophils (Fig. 3b). Of note, *R*-roscovitine at 100 mg per kg reduced the inflammatory cells to near basal numbers normally found in the naive mouse pleural cavity. Anti-inflammatory effects of *R*-roscovitine were also evident because edema formation (total exudate volume obtained by pleural



**Figure 4** Role of caspase-dependent apoptosis in *R*-roscovitine-enhanced resolution of carrageenan-induced pleurisy. (**a,b**) The caspase inhibitor zVAD-fmk prevents *R*-roscovitine-induced resolution of carrageenan-induced inflammation. Twenty-four hours after intrapleural injection of 1% carrageenan, male C57/bl6 mice were treated with 10 mg per kg of *R*-roscovitine (i.p.) and/or 5 mg per kg of zVAD-fmk (i.p. at 4-h intervals). Mice were killed 36 h after carrageenan injection and total pleural inflammatory cell numbers (**a**) and exudate volumes (**b**) were measured. Data represent the mean  $\pm$  s.e.m. (8–10 mice per group). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus DMSO control. (**c**) zVAD-fmk prevents apoptosis during *R*-roscovitine-induced resolution of carrageenan-induced inflammation. Mice were treated as in **a,b**, and apoptosis was analyzed in pleural inflammatory cells by annexin-V labeling and flow cytometry ( $n = 8$ –10 mice per group). \* $P < 0.05$  versus DMSO control. Inflammatory cell apoptosis was also assessed by morphology using cyto-centrifuge preparations stained with hematoxylin and eosin. (**d–g**) *R*-Roscovitine reduces inflammation in pleural lavage exudates and lung tissue, and decreases the numbers of macrophages containing apoptotic bodies in pleural lavage. Twenty four hours after intrapleural injection of 1% carrageenan, male C57/bl6 mice were treated i.p. with vehicle control (**d,f**) or 100 mg per kg of *R*-roscovitine (**e,g**). Mice were killed 36 h after carrageenan injection. Cyto-centrifuge preparations stained with hematoxylin and eosin (**d,e**) and tissue sections of pleural lavage and lungs (**f,g**) were assessed morphologically by microscopy (original magnification  $\times 40$ ). Asterisks indicate viable neutrophils (**d**); arrows indicate phagocytosed apoptotic neutrophils (**e**).

washouts) decreased markedly with *R*-roscovitine treatment as compared with saline control (\* $P < 0.05$ ; **Fig. 3c**). In parallel, release of the pro-inflammatory mediators IL-6 (**Fig. 3d**), IFN- $\gamma$  (**Fig. 3e**) and MCP-1 (**Fig. 3f**) in the exudate was also diminished by *R*-roscovitine.

We next determined the effect, over a period of 7 d, of administering *R*-roscovitine (100 mg per kg i.p.) at the peak of inflammation (24 h). We observed a reduction in total cell numbers in the pleural cavity at all time points (**Fig. 3g**) and in neutrophil and monocytes/macrophages numbers at 36 h after carrageenan injection (**Fig. 3a,b**), providing further evidence that this CDK inhibitor enhances inflammatory resolution. Because *R*-roscovitine induced caspase-dependent apoptosis of human neutrophils *in vitro* (**Fig. 2a,b**), we established whether the enhanced resolution of inflammation by *R*-roscovitine was mediated by a similar induction of caspase-dependent inflammatory cell apoptosis *in vivo*. Systemic administration of the broad-spectrum caspase inhibitor zVAD-fmk prevented the *R*-roscovitine-induced decrease in inflammatory cells and edema formation in the pleural cavity in carrageenan-induced pleural inflammation (**Fig. 4**). Administration of zVAD-fmk prevented the resolution of inflammation *in vivo* by increasing the total inflammatory cell number (**Fig. 4a**) and edema formation (**Fig. 4b**) as compared with saline or vehicle controls. In addition, the increase in inflammatory cell apoptosis (**Fig. 4c**) and macrophages containing apoptotic bodies (see **Fig. 4d,e**) observed with *R*-roscovitine treatment in the pleural cavity was inhibited markedly by treatment with zVAD-

fmk. We also noted that lungs of mice treated intrapleurally with carrageenan had a consequent marked inflammatory infiltrate (**Fig. 4f**), which was reduced by *R*-roscovitine treatment (**Fig. 4g**).

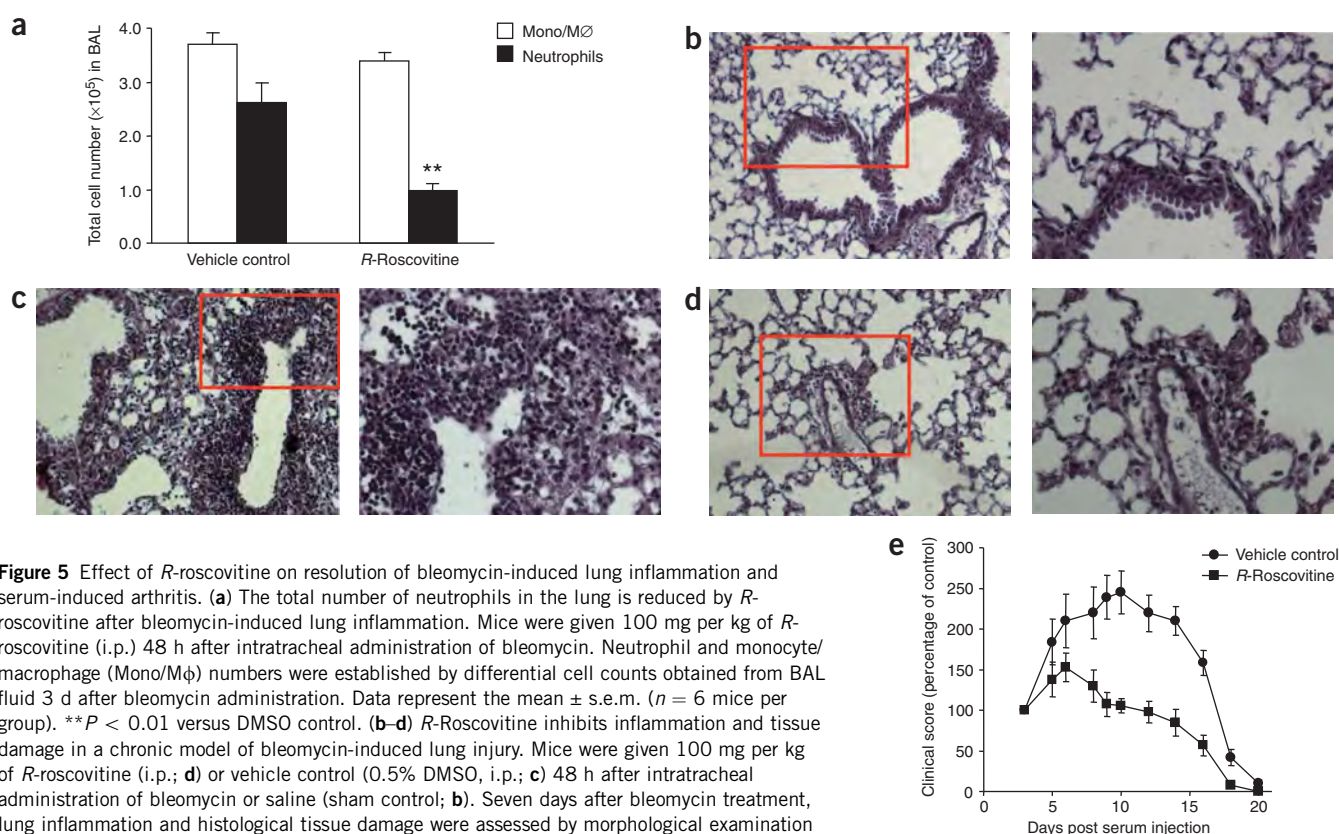
#### Roscovitine enhances inflammatory resolution in other models

The anti-inflammatory and pro-resolving effects of *R*-roscovitine were further confirmed in another two inflammatory models. In the bleomycin-induced lung injury model, *R*-roscovitine (100 mg per kg i.p.), given after inflammation was established, reduced the total number of neutrophils in the bronchoalveolar fluid 3 d after bleomycin administration (\*\* $P < 0.01$ ; **Fig. 5a**). Histological examination showed that, when bleomycin-induced lung injury was allowed to persist for 7 d, *R*-roscovitine reduced bleomycin-induced inflammation (**Fig. 5b–d**). *R*-Roscovitine, when administered with the sham intratracheal saline control, had no obvious detrimental effects on the lung pathology (**Fig. 5b**). *R*-Roscovitine also reduced bleomycin-induced lethality (3 out of 8 mice treated with bleomycin alone died by day 12, as compared with none of the *R*-roscovitine- and bleomycin-treated mice). In addition, in a model of passively induced arthritis, we observed that arthritis, as assessed by clinical scores, resolved more quickly in *R*-roscovitine-treated than in control mice (**Fig. 5e**).

#### DISCUSSION

Specific induction of inflammatory cell apoptosis represents a new approach for future treatment of inflammatory diseases. The





**Figure 5** Effect of *R*-roscovitine on resolution of bleomycin-induced lung inflammation and serum-induced arthritis. **(a)** The total number of neutrophils in the lung is reduced by *R*-roscovitine after bleomycin-induced lung inflammation. Mice were given 100 mg per kg of *R*-roscovitine (i.p.) 48 h after intratracheal administration of bleomycin. Neutrophil and monocyte/macrophage (Mono/Mφ) numbers were established by differential cell counts obtained from BAL fluid 3 d after bleomycin administration. Data represent the mean  $\pm$  s.e.m. ( $n = 6$  mice per group). \*\* $P < 0.01$  versus DMSO control. **(b–d)** *R*-Roscovitine inhibits inflammation and tissue damage in a chronic model of bleomycin-induced lung injury. Mice were given 100 mg per kg of *R*-roscovitine (i.p.; **d**) or vehicle control (0.5% DMSO, i.p.; **c**) 48 h after intratracheal administration of bleomycin or saline (sham control; **b**). Seven days after bleomycin treatment, lung inflammation and histological tissue damage were assessed by morphological examination (original magnification  $\times 40$ ) of formaldehyde-fixed lung sections stained with hematoxylin and eosin. Images are representative of six mice per group. **(e)** *R*-Roscovitine enhances the resolution of passively induced arthritis. Mice ( $n = 10$  in each group) were injected twice (days 0 and 2) with K/BxN serum derived from arthritic (day 60) K/BxN transgenic mice. Clinical scores were assessed on the days indicated. Control mice received vehicle (0.5 ml, i.p.) and treated mice received *R*-roscovitine (0.5 ml, 10 mg per kg, i.p.) on days 3, 5, 7, 9, 11, 13, 15, 17 and 19. Data represent the mean  $\pm$  s.e.m. of the clinical score obtained on day 3 before the first injection of *R*-roscovitine. \*\*\* $P < 0.001$  by two-way ANOVA.

coordinated cascade of events that follows inflammatory insult is highly regulated from onset to resolution through checkpoints that control cell migration, infiltration and survival and perpetuation of the inflammatory response. Migratory cells either die in a pre-programmed manner through apoptosis or may persist *in situ* in a potentially detrimental manner to promote tissue destruction by continued excessive secretory activity or through the consequences of cell death by secondary necrosis. The ingestion of apoptotic cells by inflammatory macrophages also promotes the synthesis and release of mediators with anti-inflammatory properties (such as TGF- $\beta$ 1 and IL-10)<sup>4,5</sup>. CDKs are essential cell signaling proteins that traditionally have been thought to control exclusively the fate of proliferating cells, in which CDK dysfunction is probably involved in increased cell turnover and tumor progression. Current therapies aimed at inhibiting CDKs are being developed for the treatment of various cancers.

Our study identifies a mechanism for both accelerating apoptosis of human neutrophils by CDK inhibitors *in vitro* and facilitating resolution of neutrophil-dependent inflammation *in vivo*. We have shown that human neutrophils possess CDK1, CDK2 and CDK5, and that specific inhibitors of CDKs (namely, *R*-roscovitine, NG75 and hymenialdisine) induce caspase-dependent apoptosis in a time- and concentration-dependent manner. The CDK inhibitors induced apoptosis even in the presence of diverse powerful pro-survival agents (dbcAMP, GM-CSF and LPS) that retard human neutrophil apoptosis through distinct molecular mechanisms. Compelling evidence suggests

that contaminating monocytes contribute to LPS-mediated neutrophil survival<sup>25,26</sup>, triggering monocyte synthesis and release of neutrophil survival factors. Regardless of the precise mechanisms of LPS action, our data clearly show that the CDK inhibitors can overcome them. Thus, the ability of CDK inhibitors to override endogenous pro-survival factors further suggests their potential use in inflammatory diseases where concentrations of such mediators are increased.

We found that the expression of CDK1 and CDK2 proteins did not change during apoptosis, but that treatment of neutrophils with the pro-apoptotic activating antibody to Fas (CH11) resulted in loss of CDK1 activity before the onset of apoptosis. By contrast, a previous study has shown that non-cycling neuronal cells require activation of CDK for the induction of apoptosis and that CDK inhibitors can prevent cell death<sup>10</sup>. Our results suggest that CDK activity is necessary and fundamental to neutrophil survival. We have also shown that *R*-roscovitine-induced apoptosis is caspase dependent and is associated with a loss of expression of the Bcl-2 family protein Mcl-1 (ref. 14). The loss of Mcl-1 caused by CDK inhibitors is probably an important mechanism controlling apoptosis<sup>11–13</sup>. In addition, because CDK5 is also a target for *R*-roscovitine<sup>16</sup> and this CDK has been reported in human neutrophils<sup>27</sup>, we confirmed that CDK5 protein is expressed in human neutrophils (data not shown). Gene array experiments have reported the expression of several CDK mRNAs, endogenous CDK inhibitors and associated proteins in neutrophils<sup>32–35</sup>, some of which are altered during apoptosis<sup>36</sup>.

We previously showed that inhibiting pro-survival molecules from the MAPK and Bcl-2 families (namely, ERK1/2 and Bcl-x<sub>L</sub>) promotes resolution of inflammation by inducing inflammatory cell apoptosis in an acute rat model of carrageenan-induced pleurisy, whereas inhibition of the pro-apoptotic molecule Bax prevents resolution of inflammation<sup>30</sup>. We have now shown that *R*-roscovitine promotes resolution of inflammation in carrageenan-induced pleurisy in a dose-dependent manner. *R*-Roscovitine induced apoptosis *in vivo*, as assessed by annexin-V labeling and morphological analysis. *R*-Roscovitine also inhibited the release of pro-inflammatory cytokines, including IL-6, MCP-1 and IFN- $\gamma$ , further supporting its pro-resolution role. In addition, *R*-roscovitine, administered at the peak of inflammation, enhanced the resolution of bleomycin-induced lung inflammation. This model was used for its clinical relevance<sup>37</sup> and because progression of the marked acute inflammatory response induced by bleomycin leads to chronic inflammation and fibrosis<sup>38,39</sup>. Early accumulation of neutrophils in the BAL fluid and subsequent lung inflammation and injury were attenuated by *R*-roscovitine treatment. *R*-Roscovitine also reduced bleomycin-induced lethality, indicating that CDK inhibition attenuates prolonged inflammation, leading to lung damage and consequent death. *R*-Roscovitine also caused enhanced inflammatory resolution in serum-induced arthritis as assessed by improved clinical scores.

Our *in vitro* work showing that the CDK inhibitors promote neutrophil apoptosis adds to the evidence indicating that neutrophils are essential in regulating the inflammatory responses *in vivo*, including models of carrageenan-induced pleurisy<sup>30</sup>, bleomycin induced-lung injury<sup>38,39</sup> and arthritis<sup>7</sup>. We also showed that inhibition of apoptosis with zVAD-fmk prevents the enhanced resolution of inflammation by *R*-roscovitine in carrageenan-induced pleurisy. The dosing regime for zVAD-fmk administration is paramount to elicit an effect *in vivo* and was based on studies where injections were administered every 3 h (ref. 40). Notably, *R*-roscovitine-induced apoptosis and its anti-inflammatory effects were reversed *in vivo* by the caspase inhibitor zVAD-fmk, providing further evidence that the anti-inflammatory mechanism of the CDK inhibitor is due to the induction of caspase-dependent inflammatory cell apoptosis.

Although neutrophils are terminally differentiated, evidence indicates that during the early stages of resolution of inflammation they are capable of phenotypic alteration, switching from a phenotype that generates pro-inflammatory mediators to one generating mediators that are anti-inflammatory and/or pro-resolving. For example, neutrophils can produce and respond to agents, such as lipoxins and resolvins, that have anti-inflammatory and pro-resolution properties<sup>41–43</sup>. In addition, accelerated resolution of inflammation has been reported for endogenous lipid mediators such as protectin D1 (ref. 44), suggesting that control of inflammation can be achieved physiologically through production of endogenous mediators. Our hypothesis that manipulation of pro-resolution mechanisms can have profound effects on established inflammation is strongly supported by data showing that pharmacological manipulation of apoptosis and/or macrophage clearance can be achieved<sup>30,45</sup> and our present finding that modulation of granulocyte apoptosis with CDK inhibitors promotes resolution of inflammation.

Thus, systemic administration of a specific inhibitor of CDKs induces apoptosis of inflammatory cells *in situ* and promotes inflammatory resolution. *In vivo* studies investigating the effect of CDK inhibition in cancer have shown that systemic administration of *R*-roscovitine reduces tumor size<sup>46</sup> and that *R*-roscovitine (even at high doses of 2 g per kg) is well tolerated. It is widely acknowledged that tumor cells have defective CDK pathways that promote cell proliferation and

prevent cell apoptosis<sup>8,9</sup>, suggesting that CDK inhibitors specifically target these pathways to elicit their therapeutic action. Our study has shown that CDK inhibitors also promote apoptosis in non-proliferating inflammatory cells *in vitro* and accelerate inflammatory resolution by promoting apoptosis and subsequent safe clearance of neutrophils by macrophages *in vivo*. In conclusion, given the renewed interest in the close association between inflammatory processes and cancer<sup>47,48</sup> and the potential problems with using monoclonal antibodies and other biological agents, this newly identified role of the small-molecule anti-cancer CDK inhibitors may have potential for the treatment of diseases associated with increased or persistent inflammatory responses.

## METHODS

**Neutrophil isolation and assessment of apoptosis.** We isolated human blood neutrophils<sup>23,49</sup>, a process that takes ~2 h, and cultured the cells ( $5 \times 10^6$ /ml, 37 °C) in Iscove's modified Dulbecco's medium (IMDM) containing 10% autologous serum. The cells were incubated with the following reagents: *R*-roscovitine, (*R*)-2-[[9-(1-methylethyl)-6-[(phenylmethyl)amino]-9H-purin-2-yl]amino]-1-butanol (A.G. Scientific); hymenialdisine, 4-(2-amino-4-oxo-2-imidazolidin-5-ylidene)-2-bromo-4,5,6,7-tetrahydropyrrolo[2,3-c]azepin-8-one (from L. Meijer, Roscoff, France); NG75, 2-(1*R*-isopropyl-2-hydroxyethylamino)-6-(4-methoxy-benzylamino)-9-isopropylpurine (from N. Gray, University of California, Berkeley); db-cAMP (Sigma); GM-CSF (R&D Systems); and LPS (*Escherichia coli* 0127:B8, Sigma). We assessed apoptosis by flow cytometry using annexin-V-conjugated fluorescein isothiocyanate (FITC; Roche) in combination with PI (Sigma) and used light microscopy to confirm the presence of morphological changes characteristic of apoptosis<sup>2,23</sup>.

**Western blotting.** We lysed cells ( $5 \times 10^6$ ) for 15 min with 0.1% Nonidet P40 in 1 ml of TBS containing a protease inhibitor cocktail<sup>23</sup> before centrifugation (23,100g, 4 °C, 20 min). Protein samples (40  $\mu$ g or the equivalent of  $1.5 \times 10^6$  cells per lane) were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. Blots were blocked with 5% skimmed milk powder in TBS plus Tween before probing with antibodies to CDK1 (Transduction Laboratories), CDK2 (Transduction Laboratories), CDK5 (Santa-Cruz Biotechnology), caspase-3 (Cell Signaling Technologies), Mcl-1 (Santa-Cruz Biotechnology) or  $\beta$ -actin (Sigma).

**Kinase assays.** We assayed kinase activity by immunoprecipitation of CDK1 (A17 antibody; Pharmingen) or CDK2 (M2 antibody; Santa Cruz Biotechnology), followed by incubation with histone H1 and [ $\gamma$ -<sup>32</sup>P]ATP (ref. 50). Immunoprecipitates were resolved by SDS-PAGE, stained with Coomassie blue to visualize the histone H1 bands, dried and exposed for autoradiography.

**Carrageenan-induced pleurisy.** Male C57BL/6J mice (aged 8–12 weeks) were injected intrapleurally with 0.1 ml of 1%  $\lambda$ -carrageenan (Marine Colloids), followed 24 h later by 0.5 ml of saline control, 0.5 ml of 0.5% DMSO vehicle control, or 10 or 100 mg per kg of *R*-roscovitine (i.p.). Mice were killed and pleural cavities were lavaged with 1 ml of 3.15% (w/v) sodium citrate in PBS. Edema formation was measured by pleural exudate weight. Cell counts, apoptosis and macrophage phagocytosis in pleural cell samples were assessed microscopically.

**Administration of zVAD-fmk.** Twenty-four hours after intrapleural injection of carrageenan, mice were injected i.p. with 10 mg per kg of *R*-roscovitine and/or 5 mg per kg of zVAD-fmk (z-Val-Ala-DL-Asp-fluoromethylketone; Bachem) at a final concentration of 1 mg/ml in saline, or with appropriate amounts of DMSO vehicle control. Two additional doses of zVAD-fmk were given i.p. 4 and 8 h later, and all mice were killed after 12 h.

**Cytokine analysis.** We measured cytokine levels by using a mouse inflammation cytokine bead kit (BD Biosciences) according to the manufacturer's instructions.

**Bleomycin-induced lung injury.** Male C57BL/6J mice (aged 8–12 weeks) were given either 0.05 ml of 0.1 U of bleomycin (Apollo Scientific) or saline intratracheally (sham control), and then treated 48 h later with either 0.5 ml of 0.5% DMSO vehicle control or 100 mg per kg of *R*-roscovitine. The mice were killed 72 h or 7 d after bleomycin or saline administration. In the acute

72-h experiments, bronchoalveolar lavage were performed with three sequential washes with 0.8 ml of ice-cold saline before perfusion with 4% formaldehyde for tissue histological analysis. Differential cell counts were done on cytocentrifuge preparations with eosin and hematoxylin staining. Histological analysis of the 7-d experiments was done without bronchoalveolar lavage to maintain tissue integrity. Experiments were done on untreated control, sham control (saline and DMSO treatment), R-roscovitine control (saline and 100 mg per kg of R-roscovitine), bleomycin plus vehicle (DMSO) control, and bleomycin plus R-roscovitine treatment groups, with six mice per group. Lungs were inflated, fixed with 1 ml of 4% formaldehyde and then decalcified with 5% nitric acid for 3 h, and lung injury was assessed by histological examination of paraffin-embedded lung sections stained with hematoxylin and eosin.

**Induction and assessment of arthritis.** Female C57BL/6J mice (aged 6–8 weeks) received two i.p. injections at 48-h intervals of sera (100 µl) from arthritic K/BxN mice (aged 60 d) and R-roscovitine or vehicle was administered i.p. every 48 h. Arthritis was scored by clinical examination: a score of 1 represents erythema alone or swelling of 1 digit; 2 represents erythema plus swelling of the tarsal joints, or swelling of the hock or wrist joint alone; 3 represents swelling in both tarsal and hock joints, both wrist and digits, or more than two digits and the tarsal joints. The mean cumulative limb scores for each mouse were calculated for each group, and data are expressed as the percentage of the clinical score obtained on day 3 before the first injection of R-roscovitine.

**Statistical analysis.** We analyzed *in vitro* experiments by analysis of variance (ANOVA) and Student-Newman-Keuls comparison tests. All *in vivo* experiments included at least six mice per group and experiments were repeated to verify the original findings. Statistical analysis was done by a one-way ANOVA with a Bonferroni multiple comparison *post hoc* test with a 95% confidence interval. Data are expressed as the mean ± s.e.m. and values of  $P < 0.05$  were considered significant.

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#### AUTHOR CONTRIBUTIONS

A.G.R. initiated, designed, directed and performed experiments and took overall responsibility for planning and writing the manuscript. D.A.S. helped design, perform and analyze the *in vivo* experiments and contributed to the writing of the *in vivo* component. A.W. helped design, perform and analyze the *in vitro* experiments and contributed to the writing of the *in vitro* component. C.W. contributed intellectually to the manuscript and helped with the *in vitro* work. T.A.S. performed some *in vitro* apoptosis and western blotting experiments. N.A.R. performed the Mcl-1 western blot experiments. A.C. and M.M.-L. performed some *in vitro* apoptosis experiments. T.R.W. helped in the design of the western blotting and kinase experiments. R.D. helped in the bleomycin experiments. M.G. helped in the design and execution of the arthritis experiments. E.C. helped in the design and execution of the kinase experiments. M.C.M. performed preliminary apoptosis experiments with the CDK inhibitors. H.J.B. provided intellectual input and provided significant input to the design and execution of the kinase experiments. J.S.S. provided intellectual input. I.D. provided intellectual input and contributed to the design of the experiments and the writing of the manuscript. C.H. provided intellectual input and helped in the design and coordination of the project.

#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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## Corrigendum: Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis

Adriano G Rossi, Deborah A Sawatzky, Annemieke Walker, Carol Ward, Tara A Sheldrake, Nicola A Riley, Alison Caldicott, Magdalena Martinez-Losa, Trevor R Walker, Rodger Duffin, Mohini Gray, Elvira Crescenzi, Morag C Martin, Hugh J Brady, John S Savill, Ian Dransfield & Christopher Haslett

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In the version of this article initially published, the dose stated for zVAD-fmk administration was incorrect. The methods reported on page 1062 should read, "Twenty-four hours after intrapleural injection of carrageenan, mice were injected i.p. with 10 mg per kg of R-roscovitine and/or 5 mg per kg of zVAD-fmk (z-Val-Ala-DL-Asp-fluoromethylketone; Bachem)." Similarly, the legend to Figure 4, line 3, should read "C57/bl6 mice were treated with 10 mg per kg of R-roscovitine (i.p.) and/or 5 mg per kg of zVAD-fmk (i.p. at 4-h intervals)." The authors also made an error reporting the time of administration of K/BxN serum in the legend to Figure 5, line 14. This should read "Mice ( $n = 10$  in each group) were injected twice (days 0 and 2) with K/BxN serum derived from arthritic (day 60) K/BxN transgenic mice." The error has been corrected in the HTML and PDF versions of the article.



## Granulocyte Apoptosis and Macrophage Clearance of Apoptotic Cells as Targets for Pharmacological Intervention in Inflammatory Diseases

Nicola A. Riley, Carol Ward, Deborah A. Sawatzky, Tara A. Sheldrake, Ian Dransfield, Christopher Haslett and Adriano G. Rossi\*

MRC Centre for Inflammation Research, The Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK

**Abstract:** A subset of leukocytes, known as the granulocytes, are the body's first line of innate immune defense. The granulocytes are comprised of neutrophils, eosinophils and basophils of which the former two will be the focus of this review. Neutrophils defend the body against bacterial and fungal infection whilst eosinophils are thought to defend against parasitic invasions. Granulocytes are recruited to the site of infection or tissue damage where their relatively short half-life can be extended by regulatory external factors including hypoxic environments or agents that activate signaling pathways, such as NF- $\kappa$ B which is implicated in the up-regulation of anti-apoptotic genes. Granulocytes release various proteins, proteolytic enzymes and toxic oxygen products into the phagolysosome or surrounding environment destroying the invading organism. However, in order for inflammation to be resolved it is essential that granulocytes die by apoptosis and are phagocytosed by macrophages in a non-inflammatory fashion. This prevents the release of the cell's histotoxic contents into the extracellular milieu thereby reducing the potential for tissue damage. In instances when granulocytes fail to appropriately enter apoptosis or a defect in phagocytic clearance occurs the inflammatory response can be perpetuated, potentially resulting in the development and promotion of inflammatory disorders such as asthma or rheumatoid arthritis. Thus, selective enhancement of apoptosis and augmentation of macrophage clearance could allow targeting of inflammatory resolution to provide potential novel therapeutic agents for the treatment of inflammatory disorders.

### INTRODUCTION

Neutrophil, eosinophil and basophil leukocytes are collectively known as granulocytes and are the main cells involved in innate immune defense. Neutrophils make up the highest proportion of the leukocytes in the circulation (approximately 50 - 70%) defending the body against bacterial and fungal infections. Eosinophils, on the other hand, usually comprise only 1-4% of the circulating leukocytes with their main function being to defend against parasitic infection. Basophil granulocytes are numerically very scarce (< 1% of circulating leukocytes), are thought to be involved in parasitic infection and allergic disease, and can undergo regulated cell death by apoptosis, however these cells will not be the focus of this review (see [1,2] for excellent reviews). Granulocytes derive their name from their abundant cytoplasmic granules that contain toxic agents (e.g., proteases) which play a pivotal role in the innate immune response. In response to the presence of foreign organisms or tissue damage, granulocytes and mononuclear cells are recruited to the site of damage. Here the granulocytes destroy and eliminate invading organisms by releasing bactericidal proteins, proteolytic enzymes and toxic oxygen products into the phagolysosome or surrounding environment, where they bring about the destruction of the invading microorganism. In order for the inflammation to be resolved, the granulocytes must undergo apoptosis; a highly regulated physiolo-

gical form of cell death that results in loss of responsiveness to external stimuli and ultimate functional shut-down [3-5]. Apoptosis, rather than necrosis, is essential as the cellular membrane of the granulocytes remain intact throughout therefore preventing the uncontrolled release of their histotoxic contents into the extracellular milieu. Secondly apoptotic granulocytes are rapidly recognized and removed from the site of inflammation via phagocytosis by neighboring cells or macrophages using mechanisms that dampen inflammation [3,6-8]. Failure of this process causes granulocytes to undergo necrosis where the cell membrane ruptures allowing the cytotoxic contents to be released into the extracellular milieu resulting in damage to surrounding tissue and an exacerbation of the inflammatory process. Thus, failure of granulocytes to appropriately enter apoptosis at a suitable rate or, alternatively, a defect in phagocytic clearance of the apoptotic granulocytes can lead to a disrupted inflammatory response. In such instances this could prevent effective resolution of inflammation resulting in development or promotion of various inflammatory disorders. The granulocytes have therefore been implicated in diseases where inflammatory resolution is disrupted. For example, eosinophils are involved in allergic type diseases (e.g., asthma and allergic rhinitis) and neutrophils are involved in diseases such as rheumatoid arthritis and inflammatory bowel disease. Therefore, strategic use of therapeutic agents that increase selective granulocyte apoptosis in conjunction with those that up-regulate macrophage phagocytosis, could pose a realistic approach to developing novel anti-inflammatory treatments [9-11].

\*Address correspondence to this author at the MRC Centre for Inflammation Research, The Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK; Tel: +44-131-242-6659; Fax: +44-131-242-6578; E-mail: a.g.rossi@ed.ac.uk



## STRUCTURAL AND CELLULAR CHANGES OCCURRING DURING APOPTOSIS

Apoptosis is an evolutionary conserved pathway and like virtually all cells granulocytes undergo this form of programmed cell death [12]. Granulocytes having left the bone marrow enter the circulation (it is estimated that the half life of neutrophils in the circulation is approximately 6 hours) and in response to appropriate stimuli enter the tissues. Once their physiological role (e.g., killing and digestion of invading organisms) has been accomplished the cells are destined to undergo spontaneous or constitutive apoptosis in the tissues. Isolated human neutrophils under optimal *in vitro* culture conditions can survive up to 20-40 hours whereas eosinophils *in vitro* tend to survive longer (40-80 hours) before apoptosis is engaged. Apoptosis can be delayed significantly by a number of environmental factors and mediators. However, once the process of apoptosis has been initiated (usually by the activation of caspases – see below) there is a reduction in granulocyte volume, their chromatin condenses and their DNA is cleaved internucleosomally. Most cell types fragment, resulting in the release of many membrane encapsulated apoptotic bodies; however granulocytes tend to stay intact resulting in the formation of relatively few apoptotic bodies per cell. The apoptotic bodies are membrane enclosed thereby preventing release of the intracellular contents into the extracellular milieu, and preventing damage to neighboring cells and tissues. Apoptotic cells are then rapidly recognized and engulfed by phagocytes (e.g., macrophages) in response to the exposure of phagocytosis signals (e.g., phosphatidylserine (PS)) on the surface of the apoptotic cell [8,13]. Manipulation of this process therefore poses huge potential for the treatment of inflammatory disorders. However in order for this to occur it is essential that every aspect of apoptosis from cell type specific variations to general regulatory mechanisms are fully understood.

## APOPTOTIC PATHWAYS

There are a number of signaling pathways that can regulate the rate of apoptosis in granulocytes, like most other cells, apoptosis can be initiated *via* two conserved pathways, commonly known as the extrinsic or the intrinsic pathways.

### EXTRINSIC PATHWAY

The extrinsic pathway triggers apoptosis through the stimulation of cell surface 'death receptors' for example TNFR, Fas, and TRAILR (all of which are present on human neutrophils [14] and eosinophils [15]) by specific counter ligands. Interestingly the ligands for these receptors not only initiate apoptotic pathways but can also impact upon other signaling pathways that influence cellular responsiveness. The role of TNF $\alpha$  receptors in neutrophils is particularly notable as they have the capacity to 'prime' the cells for subsequent enhanced stimulation by other agonists (e.g., formylated peptides) and infer both pro-apoptotic and anti-apoptotic signaling depending upon the length of the incubation period with TNF $\alpha$ . Neutrophils cultured in the presence of TNF $\alpha$  for 12 hours or longer has been reported to have a delayed rate of apoptosis whilst conversely periods of culture of 8 hours or less appear to trigger apoptosis in subpopulations of neutrophils [16]. The TNF $\alpha$  pro-apoptotic and

anti-apoptotic pathways are activated upon binding of TNF $\alpha$  to TNFR1 (TNF receptor 1) and TNFR2 [16,17]. The receptors trimerise inducing proximity of their death domains, consequently allowing the death domains of TNFR-associated death domain-containing proteins (TRADDs) to bind. TRADD can also induce apoptosis through binding to FADD subsequently activating pro-caspase-8 and has the ability to activate the transcription factors NF- $\kappa$ B and AP-1 *via* binding to secondary adaptor molecules such as TNFR-associated factor-2 and Receptor-Interacting Protein (RIP), which may implicate TNF $\alpha$  as also having a role in protecting the cell from apoptosis as NF- $\kappa$ B is known to induce anti-apoptotic factors.

Activation of Fas is also thought to stimulate dual properties within neutrophils, with membrane bound FasL being postulated to be a more effective inducer of apoptosis than soluble FasL. Soluble FasL (sFasL) may play a more pivotal role in cell migration as it has been exhibited to possess chemotactic properties, which are thought to be due to activation of signaling pathways separate to those involved in the induction of apoptosis [14]. Once activated Fas receptors trimerise allowing interaction with the FADD adapter protein and pro-caspase-8 creating the Death Initiating Signaling Complex (DISC) to yield active caspase 8 through induced proximity, which then initiates the cascade of caspase activation, activating either pro-caspases 3, 6 or 7. The binding of c-FLIP a competitive inhibitor of pro-caspase-8, can inhibit the activation of the DISC complex. Similarly ligation of Fas on eosinophils leads to enhanced rates of apoptosis [10,18].

TRAIL and multiple TRAIL-receptors (TRAIL-R) 1-4 have been identified as cell surface death receptors. So far it is known that human neutrophils express TRAIL, TRAIL-R2 and TRAIL-R4. Of these TRAIL receptors, TRAIL-2 has been identified as having apoptosis inducing ability in some cell types as it contains a death domain, however TRAIL-4 lacks a death domain rendering it incapable of inducing apoptosis. As a result of this it is hypothesized that TRAIL-4 acts as a decoy receptor with anti-apoptotic capacity. However, TRAIL is thought not to be involved in the regulation of constitutive apoptosis but instead in the removal of neutrophils from inflammatory sites [14,15,19,20]. Interestingly TRAIL-R1, 3 and 4 have been found on purified human eosinophils and surprisingly stimulation of eosinophils by TRAIL caused survival in some cells using undefined mechanisms [15].

### INTRINSIC PATHWAY

Cellular stress or injury triggered by, for example, UV irradiation or withdrawal of growth or survival factors activates the intrinsic apoptotic pathway. In this instance the key players in the initiation of apoptosis are a family of proteins called the Bcl-2 family (see below), which control the release of cytochrome C from the mitochondria by increasing outer membrane permeability. This allows the release of pro-apoptotic factors from the intermembrane space into the cytoplasm; for example, cytochrome C release is responsible for the formation of the apoptosome (a complex comprising apoptosis protease activating factor-1 (Apaf-1), caspase-9, caspase-3), Smac/Diablo and Htra2/Omi, which obstruct the



Inhibitor of Apoptosis (IAP) family of caspase inhibitors, Alt/endonuclease G which are important in caspase-independent nuclear changes in apoptosis and Heat Shock protein 60 [21].

### THE BCL-2 FAMILY – REGULATORS OF APOPTOSIS AND THE ROLE OF THE MITOCHONDRIA

Members of the Bcl-2 family are a series of cell death regulatory proteins, of which there have been over 20 members identified so far [22]. They are divided into three subgroups depending upon their function and the number of Bcl-2 Homology (BH) domains they contain, of which there are four in total. These subgroups are the anti-apoptotic Bcl-2 family and the pro-apoptotic family, which is further subclassified into multi-domain pro-apoptotic Bcl-2 members and BH3 domain only pro-apoptotic Bcl-2 members. Neutrophils and eosinophils are known to contain various members of the Bcl-2 family which play an essential role in regulating their apoptotic processes. For example, neutrophils constitutively express multi-domain pro-apoptotic members Bax and Bak and the BH3 only domain pro-apoptotic members Bid and Bad [23]. Bax, which has a relatively long half life, undergoes translocation to the mitochondria during constitutive apoptosis resulting in the activation of the intrinsic apoptotic pathway culminating in the activation of caspase 3 [24-28]. The translocation of Bax is thought to be caspase independent due to its non-inhibition by the broad range caspase inhibitor z-VAD-fmk. Furthermore, it is proposed that Bid, which also has a long half life, also undergoes translocation to the mitochondria where it is truncated *via* the caspases [29,30]. As previously mentioned cytochrome C is an essential molecule in the apoptotic process due to its necessity for the formation of the apoptosome (cytochrome C however remains hard to detect in neutrophils). Yet neutrophils are known to contain some (albeit relatively few) mitochondria which are a major source of cytochrome C. The key role of mitochondria in both neutrophils and eosinophils appears to be in regulation of apoptosis as opposed to respiration [29,31-37].

Anti-apoptotic members of the Bcl-2 family are also present in granulocytes. Neutrophils, whilst lacking Bcl-2, are known to express the Bcl-2 homologue A1 and the anti-apoptotic proteins Mcl-1, Bcl-X<sub>L</sub> and X-IAP. The best studied of these proteins in neutrophils is Mcl-1. Mcl-1 protein levels in blood circulating neutrophils decrease preceding the cell entering apoptosis. Inflammatory mediators that postpone neutrophil apoptosis do this (at least in part) through maintaining or increasing levels of Mcl-1 and/or A1 enabling neutrophils to carry out their vital defensive functions. The half lives of both A1 and Mcl-1 mRNA along with the Mcl-1 protein are known to be short in contrast to the long half lives of the pro-apoptotic Bcl-2 members. This offers an explanation as to why neutrophils are 'primed' for apoptosis when in the circulation as *de novo* synthesis of Mcl-1 and A1 is absent, therefore allowing the effect of long lived pro-apoptotic proteins to pre-dominate [23,38,39]. Survival signals in the form of cytokines and other mediators found at sites of inflammation induce *de novo* synthesis of the anti-apoptotic proteins, demonstrated through the use of agents that inhibit protein synthesis (e.g., cycloheximide and actinomycin D) preventing increased granulocyte survival

and directly inducing apoptosis [40,41]. One such cytokine known to increase neutrophil survival that is found at elevated levels at sites of inflammation is granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is known to increase levels of Mcl-1 protein through stimulating transcription of the Mcl-1 gene [38]. However this may not be the sole way through which GM-CSF influences Mcl-1 levels. Interestingly, it has also been shown that GM-CSF increases the cellular concentration of Mcl-1 in neutrophils to a more significant level than those achieved through enhanced *de novo* synthesis of the Mcl-1 protein by increasing the stability of the Mcl-1 protein and reducing the rate with which it is turned over via the proteasome [38].

X-IAP, a member of the inhibitor of apoptosis (IAP) family, inhibits caspases directly [42-44]. X-IAP is known to inhibit caspase 3, 7 and 9, and like Mcl-1 and A1, levels of X-IAP decrease in the presence of stress inducing and pro-apoptotic stimuli. It has been proposed by Henson *et al.* that in neutrophils activation of the ERK/MAPK pathway can protect against stress induced apoptosis by preventing a decrease in levels of X-IAP (either through inhibiting degradation or inducing X-IAP synthesis). In addition, oxidants found at sites of inflammation are hypothesized to block ERK activity *via* activation of P38, which promotes a decrease in X-IAP thereby enhancing apoptosis [45].

### CYSTEINE-DEPENDENT ASPARTATE-SPECIFIC PROTEASE FAMILY (CASPASES)

As has been mentioned above much of the initiation and execution of apoptosis is mediated via the caspase family of enzymes. The caspases are a family of death promoting proteolytic enzymes that become activated through the cleavage of their precursor molecule to generate an active caspase during apoptosis. They derive their name from ubiquitously contained cysteine residues within their active sites, which upon activation of the enzyme, cleave aspartic acid residues in their target proteins eliciting a cascade of destruction that ultimately brings about the demise of the cell. Caspases involved in apoptosis can be divided into two classes: initiator caspases and effector caspases. Initiator caspases (caspases 8, 9 and 10) as their name suggests bring about the initiation of the extrinsic (e.g., death receptor) apoptotic pathway. Pro-caspases 8 and 10 do this *via* binding to adaptor molecules for example Fas-associated death domain (FADD) using their long death effector domain (DED) containing prodomains. In addition initiator caspases can be activated by autocatalysis, which is also dependent on their prodomains, as in this instance they act to bring about the oligomerization of the initiator pro-caspases aiding autoactivation. Consequently, initiator caspases can proceed to activate the downstream effector caspases (caspases 3, 6 and 7) *via* cleavage of the effector procaspases resulting in morphological changes. Neutrophils and eosinophils have been shown to possess both initiator and effector caspases [46,47].

### CROSS TALK BETWEEN INTRINSIC AND EXTRINSIC PATHWAYS

Although in most circumstances the two pathways described above occur independently, they can be integrated by the pro-apoptotic Bcl-2 family member Bid. The crosstalk

phenomenon is initiated via caspase 8 cleaving Bid to form truncated Bid (tBid) which then translocates to the mitochondria increasing the permeability of its outer membrane resulting in the release of cytochrome C. Cytochrome C together with Apaf-1 forms the apoptosome resulting in induced proximity of procaspase-9 molecules. This results in activation of caspase-9 to activate procaspase-3 resulting in the cleavage of substrate proteins, inducing the morphological changes associated with apoptosis. Cells in which cross talk occurs are referred to as type II cells (in type I cells caspase-8 directly cleaves pro-caspase 3) and it has been proposed that neutrophils fall within the type II classification.

### INTRACELLULAR SIGNALING AFFECTING GRANULOCYTE SURVIVAL

Whilst the basic structural and cellular characteristics occurring during apoptosis vary only slightly between cell types, the mechanisms controlling apoptosis differ greatly. There are key differences in the mechanisms regulating lymphocytes and granulocytes and even between types of granulocytes [9,10]. It has been revealed through *in vitro* observations that inflammatory stimuli such as IL-1 $\beta$ , IL-2, IL-8 IL-15, TNF $\alpha$  (>12hr incubation), IFN- $\gamma$ , GM-CSF, G-CSF, LPS, C5a and hypoxic conditions all have the ability to delay neutrophil apoptosis. These pro-survival factors allow neutrophils the opportunity to persist at the site of infection ensuring efficient removal of invading pathogens. GM-CSF also acts to delay eosinophil apoptosis, while IL-5 another suppressor of eosinophil apoptosis has no effect on neutrophil life span [10]. The fate of the granulocyte at the inflammatory site can therefore be viewed in terms of a fine balance between pro-apoptotic and the anti-apoptotic signals from local pro-inflammatory mediators. Some of the pathways known to influence apoptosis will be considered.

### NUCLEAR FACTOR- $\kappa$ B (NF- $\kappa$ B)

The transcription factor NF- $\kappa$ B is a key component in regulating the fate of the granulocyte as it is implicated in up-regulating anti-apoptotic genes. It is through NF- $\kappa$ B that pro-inflammatory mediators such as LPS and TNF $\alpha$  are thought to extend neutrophil survival. The role of NF- $\kappa$ B on neutrophil and eosinophil survival has been investigated using pharmacological tools such as the NF- $\kappa$ B inhibitor gliotoxin, which when used in conjunction with exposure to LPS, was found to inhibit the anti-apoptotic effects of LPS. Additionally, at early time points gliotoxin dramatically enhances the apoptotic action of TNF $\alpha$  [48]. The importance of NF- $\kappa$ B in neutrophil and eosinophil survival has also been demonstrated using physiological NF- $\kappa$ B inhibitors, namely the prostaglandin D<sub>2</sub> metabolites  $\Delta^{12}$  prostaglandin J<sub>2</sub> ( $\Delta^{12}$ PGJ<sub>2</sub>) and 15-deoxy- $\Delta^{12,14}$  prostaglandin J<sub>2</sub> (15dPGJ<sub>2</sub>) [49]. These also increased granulocyte apoptosis and prevented mediator induced survival. In addition PGD<sub>2</sub> metabolites have been shown to possess therapeutic benefits for the treatment of rat pleurisy where they were found to induce neutrophil and macrophage apoptosis to bring about resolution of acutely inflamed tissue [50]. Although less is known about the pathways mediating eosinophil apoptosis, NF- $\kappa$ B is thought to be involved [48,51,52]. When incubated with TNF $\alpha$ , eosinophils appear to lose cytoplasmic I $\kappa$ B $\alpha$  allow-

ing the released NF- $\kappa$ B to travel to the nucleus. In addition, there is evidence suggesting that TNF $\alpha$  triggered eosinophil apoptosis may be more sensitive to NF- $\kappa$ B inhibition than constitutive apoptosis [52]. Recently, using a cell permeable HIV-tat linked super-repressor of the NF- $\kappa$ B inhibitor molecule I $\kappa$ B $\alpha$  we have confirmed a role for NF- $\kappa$ B in the regulation of eosinophil apoptosis as this inhibitor induced direct eosinophil apoptosis [51]. Other groups have now verified the importance of NF- $\kappa$ B activation in the regulation of human granulocyte apoptosis [53-58].

However, there is a dichotomy in terms of a role for NF- $\kappa$ B in inflammation where NF- $\kappa$ B also can have an anti-inflammatory/pro-resolving role during the resolution of acute inflammation [59]. The development of therapeutics that specifically manipulate the anti-inflammatory effects of NF- $\kappa$ B are currently being investigated. Although I $\kappa$ B $\alpha$  is an endogenous negative regulator of NF- $\kappa$ B, therapeutic administration of this molecule itself would not be suitable due to its rapid biodegradation *in vivo*. However, recent studies have shown that a stable chimeric form of this molecule, comprising the super-repressor I $\kappa$ B $\alpha$  fused to a membrane-transducing domain of HIV-tat protein, can promote the resolution of inflammation in an acute rat carrageenan-induced pleurisy model [60]. Furthermore, IKK $\alpha$ , an upstream regulator of I $\kappa$ B $\alpha$  and NF- $\kappa$ B, has recently been shown to negatively regulate macrophage activation, local inflammation and innate immunity to bacterial infections [61]. IKK $\alpha$  inhibits NF- $\kappa$ B activity by inducing the NF- $\kappa$ B subunits RelA and c-Rel and suppressing their interaction with pro-inflammatory gene promoters. In addition, genetically modified mice that have inactivated IKK $\alpha$  have an exaggerated inflammatory response in a model of LPS-induced septic shock [61].

### PHOSPHOINOSITIDE 3-KINASE (PI3K)

PI3K is another vital signaling molecule that is likely to regulate many of the pro-inflammatory and anti-apoptotic pathways triggered by the inflammatory mediators (e.g., LPS, GM-CSF and TNF $\alpha$ ). There are three classes of the PI3K family, class I, class II and class III, of which class I PI3Ks are subdivided again according to the organization of their catalytic and regulatory subunits along with their mode of action into classes 1A and 1B. The class 1A p85/p110 isoform of PI3K appears to be involved in the delay of neutrophil apoptosis induced by LPS, GM-CSF and TNF $\alpha$ . However, inhibition of PI3K signaling using specific PI3K inhibitors do not influence constitutive rates of neutrophil apoptosis [62]. PI3K affects apoptosis *via* controlling the activation of Akt, NF- $\kappa$ B and the cAMP response element binding protein (CREB). For example, in PI3K $\gamma$  knockout mice translocation of NF- $\kappa$ B to the nucleus and phosphorylation of CREB were decreased. This correlated with reduced expression of the anti-apoptotic proteins Bcl-X<sub>L</sub> and Mcl-1. It was also found that Akt inactivated Bad, Forkhead and GSK-3 $\beta$  through phosphorylation, with Akt now known to be an integral enzyme in neutrophil apoptosis as it controls Mcl-1 expression through CREB as well as NF- $\kappa$ B and Bcl-X<sub>L</sub> [63]. PI3K is also implicated in the regulation of eosinophil recruitment and survival *in vivo*. Recent studies, using PI3K $\gamma$ -deficient mice and specific PI3K inhibitors, have demonstrated that PI3Ks critically regulate the recruitment and



survival of eosinophils in a model of allergic pleurisy. PI3K inhibitors administered during post antigen challenge resulted in a significant increase in apoptotic events and clearance of eosinophils. It was concluded that PI3K is necessary for maintenance of eosinophilic inflammation *in vivo* and that other isoforms of PI3K may be relevant for eosinophil recruitment [64].

### MITOGEN ACTIVATED PROTEIN KINASES (MAPK)

Upstream kinases regulate the three subfamilies of MAPKs by phosphorylation. These three types of ubiquitously expressed enzymes are p38, extracellular-signal-regulated kinases (ERKs) and stress-activated protein kinase/c-jun amino-terminal kinase (SAPK/JNK); in most cell types they play a pivotal role in the regulation of survival. Inflammatory mediators activate several MAPKs therefore it is proposed that crosstalk between MAPKs may be responsible for increasing granulocyte survival. ERK pathways are known to be activated by agents that affect granulocyte responsiveness and apoptosis (e.g., GM-CSF, LPS, IL-8, IL-15 and C5a). Using MEK inhibitors (especially PD098959) it has been suggested that ERK activation is not essential for controlling constitutive apoptosis but that it is likely to be important for maintaining survival induced by certain inflammatory mediators (e.g., GM-CSF) (see [65]).

Our recent studies have shown that the pro-survival molecules pERK1/2 and Bcl-x<sub>L</sub> are present in inflammatory cells that are predominant at the onset of inflammation and become down-regulated during the resolution phase of the inflammatory response (Sawatzky *et al.* 2005; Am.J.Pathol. (in press)). Furthermore, we have qualitatively shown that inducing granulocyte apoptosis with the ERK1/2 inhibitor PD98059 promotes the resolution of inflammation when given at the peak of the inflammatory response in a rat carrageenan-induced pleurisy *in vivo* model. This study shows that the induction of neutrophil apoptosis halts the progression of the inflammatory response and pro-actively initiates the onset of resolution.

The importance of p38 and SAPK/JNK in neutrophil survival however remains more controversial. p38 may be an important factor in stress induced apoptosis, yet is thought to have no involvement in Fas or constitutive apoptosis. In addition in GM-CSF treated neutrophils it has been reported that p38 does not play a role in increasing neutrophil survival as it fails to become phosphorylated. This is supported by the observation that inhibition of p38 by SB203580 fails to affect the anti-apoptotic capacities of GM-CSF. However a possible role for p38 in neutrophil survival could be provided by its apparent ability to phosphorylate and thereby inhibit caspases-3 and 8 [45,66-68]. It has been proposed that once the caspases become activated upon the neutrophil entering apoptosis they can result in the cleavage and subsequent degradation of ERK and p38. This cleavage of MAPKs is thought to be prevented by G-CSF therefore this could pose a possible pathway through which apoptosis is inhibited in neutrophils [69,70].

There is a lack of conclusive evidence regarding the role of JNK in neutrophil apoptosis. However, the limited data

suggest that inflammatory mediators such as LPS and TNF $\alpha$  have effects on the JNK pathway to influence function. There is some debate however as to whether neutrophil apoptosis is caused by activation of JNK or if it is in fact merely a consequence of it. This is because it has been reported that MAPK kinase kinase and MEKK-1 are cleaved when the cell undergoes apoptosis and that consequently they can activate JNK [71,72].

### CYCLIC ADENOSINE MONO PHOSPHATE (cAMP)

Granulocyte apoptosis is also delayed through an increase in cAMP brought about via some inflammatory mediators (e.g., prostaglandins) and pharmacological agents that elevate cAMP (e.g., the cell permeable analogue db-cAMP) [36,73-76]. The precise mechanism of action of cAMP mediated delay of neutrophil apoptosis remains to be elucidated. It has been suggested that this effect may be mediated by both a PKA-dependent [77,78] and PKA-independent mechanism [36]. Similarly cAMP elevation delays eosinophil apoptosis however the mechanisms by which this occurs still remains to be fully elucidated.

### CALCIUM

Use of agents (e.g. A23187 and thapsigargin) that raise cytosolic free calcium in granulocytes have shown that this important second messenger molecule not only regulates granulocyte function but also apoptosis. In neutrophils, raised cytosolic calcium levels have been found to delay apoptosis [79-81] whereas increased calcium levels in eosinophils appear to enhance rates of apoptosis [80]. The reasons for such differential regulation is currently not known and it is also apparent that there is a dissociation between raised elevation of cytosolic calcium and rates of apoptosis [81].

### GLUCOCORTICOIDS

Glucocorticoids are one of the major forms of treatment for inflammatory diseases and, whilst they prove invaluable especially in lymphocytic and eosinophilic dominant diseases such as asthma they appear to be less beneficial in neutrophil-mediated inflammation. This may be because whilst glucocorticoids act to up-regulate the phagocytic ability of macrophages [7,82,83] and increase eosinophil [84] and lymphocyte [85-87] apoptosis they extend neutrophil life span [84,88,89] therefore slowing their removal from the site of inflammation. It is plausible that the same effect may occur *in vivo*. There are two known isoforms of the glucocorticoid receptor (GR), GR $\alpha$  and GR $\beta$ . The latter of which lacks a steroid binding domain rendering it unable to bind with glucocorticoids. Whilst it is known that the predominant isoform of the glucocorticoid receptor in neutrophils is the  $\alpha$ -isoform, little is known about the glucocorticoid survival pathway in neutrophils. One theory is that the ability of glucocorticoids to induce apoptosis in a cell, revolves around the ratio of GR $\alpha$  to GR $\beta$  [90]. Interestingly it has also been shown that other steroids can also affect neutrophil apoptosis. For example, oestradiol and progesterone have an anti-apoptotic effect on neutrophils, a response that is reversed by activating the Fas pathway [91].

### NITRIC OXIDE

Nitric oxide is not only important in the processes of vasodilatation and neurotransmission as it also plays a regu-

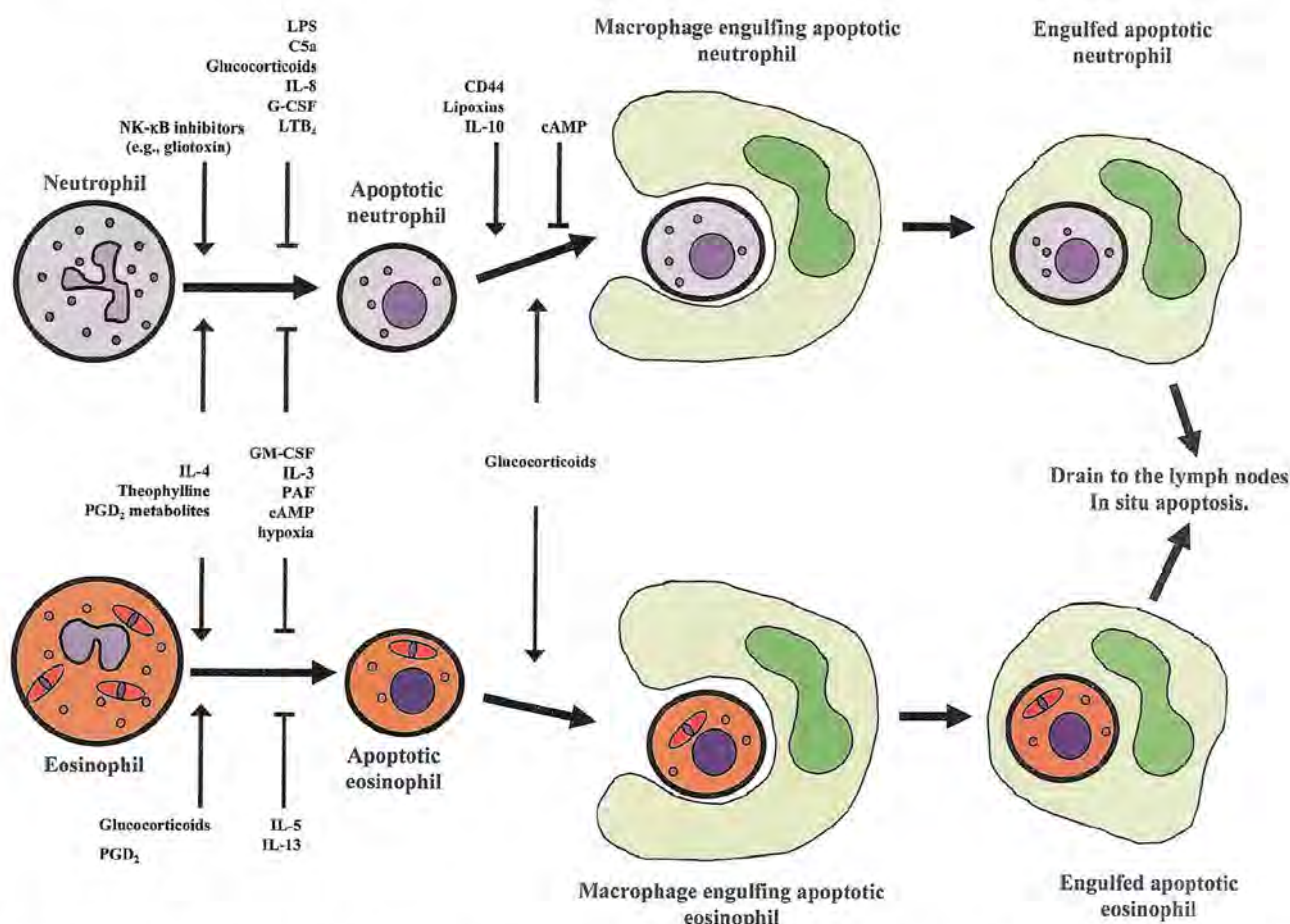


latory role in granulocyte apoptosis [92,93]. Its role however is not straightforward, as it appears to have both the ability to promote, as well as delay granulocyte apoptosis. The general trend in neutrophils however appears to be that high concentrations of nitric oxide have a pro-apoptotic/necrotic effect possibly *via* peroxynitrite generation [94]. In addition, it is speculated that pathways that influence granulocyte survival such as NF- $\kappa$ B may be disrupted indirectly by the generation of nitric oxide [95]. On the contrary, low concentrations of nitric oxide favour an anti-apoptotic effect that is proposed to involve S-nitrosation of caspase enzymes or an increase in cGMP [94].

## MISCELLANEOUS

There are many other mediators, drugs and agents that influence granulocyte apoptosis that have not been specifically covered in detail in this review. Others include, oxidative stress, bacteria and their products, and cytokines such as macrophage migration inhibitory factor (MIF), many of which have been described in other reviews [10,65,96-98]. For example, MIF an inflammatory cytokine that is released

by monocytes/macrophages T-cells and eosinophils, delays neutrophil apoptosis *via* inhibiting the apoptotic pathway upstream of the mitochondria ultimately preventing activation of caspase-3 [30]. MIF has also been proposed to be involved in eosinophil-dependent inflammatory disorders, such as asthma. Eosinophils have been shown to be an important source of MIF and bronchoalveolar lavage fluid samples from asthma sufferers demonstrating increased levels of MIF when compared to samples obtained from healthy non-asthmatics [99]. It is now recognized that the inflammatory environment is hypoxic (often with a  $pO_2 < 3\%$ ). Granulocytes function efficiently under these conditions and it is believed that adaptive responses to hypoxia are controlled mainly by the transcription factor HIF-1 $\alpha$ . Indeed it has recently been shown that HIF-1 $\alpha$  is essential for myeloid cell-mediated inflammation *in vitro* and *in vivo* [100]. Hypoxic conditions profoundly delay neutrophil [101-104] and eosinophil (Ward and Rossi, unpublished) apoptosis. The precise mechanism underlying the hypoxia-mediated neutrophil survival is unknown but good evidence suggests that HIF-1 $\alpha$ -dependent NF- $\kappa$ B activity is involved [104].



**Fig. (1).** Factors influencing human granulocyte apoptosis and macrophage clearance of apoptotic granulocytes. The life span of granulocytes is regulated at sites of inflammation by numerous factors that can increase or decrease rates of apoptosis. Some of these include agents such as GM-CSF, IL-3, PAF, cAMP and conditions such as a hypoxic environment which can delay both neutrophil and eosinophil apoptosis, whereas agents such as glucocorticoids can enhance eosinophil apoptosis but conversely delay neutrophil apoptosis. Specific agents can also act to modulate the rate at which apoptotic cells are recognized and phagocytosed by professional phagocytes. Glucocorticoids, for example, augment the ability of phagocytic removal of apoptotic cells.

## MACROPHAGE REMOVAL OF APOPTOTIC GRANULOCYTES- TARGETS FOR PHARMACOLOGICAL INTERVENTION

Resolution of inflammation requires that granulocytes are removed from the site of cellular damage by a process involving granulocyte apoptosis and subsequent disposal *via* phagocytosis by tissue resident macrophages or other cells with a capacity for phagocytosis (e.g., fibroblasts, endothelial cells). Importantly, there is much evidence suggesting that phagocytes when ingesting apoptotic cells increase their expression and release of anti-inflammatory pro-resolution mediators and down-regulate release of pro-inflammatory mediators [6,7]. Apoptotic cells experience changes to their cell membrane allowing recognition and ingestion of the apoptotic cells by phagocytes. One such signal suggested to be a key mechanism behind the removal of apoptotic cells is the recognition of PS on the apoptotic cell surface by specific PS receptors. However, PS is not thought to be the sole 'eat me' signal responsible for the removal of apoptotic neutrophils (see [8,13,105]). Therefore it has been proposed that different mechanisms may be responsible for signaling removal of apoptotic cells in different tissue types. Interestingly, it has been suggested that apoptotic cells also have the capacity to liberate specific agents that promote the recruitment of cells capable of their removal [106].

If this process is defective, it is believed that tissue damage can ensue due to the accumulation of apoptotic cells and subsequent liberation of histotoxic contents. Manipulation of the resolution phase of the inflammatory process is a promising area to provide novel anti-inflammatory therapies, selectively accelerating apoptosis in specific inflammatory cells and enhancing their subsequent clearance by exploiting their differences in intracellular regulation. Inhibition of the pro-survival signals found at the inflammatory site (for example GM-CSF and IL-5) and selective induction of specific receptors such as Fas to bring about apoptosis in chosen inflammatory cells would increase the rate of apoptosis at inflammatory sites thereby aiding the resolution process. Another optimistic area for pharmacological intervention is the regulation of the uptake of the apoptotic cells by phagocytes. It has been demonstrated that the ability of macrophages to internalize apoptotic cells can be adjusted, for example by using prostaglandins such as PGE<sub>2</sub> to elevate macrophage levels of cAMP, thereby activating PKA and transforming adhesion patterns. Macrophages treated as such have a reduced capacity to uptake apoptotic neutrophils therefore implying a possible link between adhesion and macrophage phagocytic abilities [107]. Furthermore the internalization of apoptotic neutrophils by macrophages can be upregulated via ligating the transmembrane adhesion receptor CD44 [108]. This molecule has been found to be of particular importance in the resolution of lung inflammation [109]. Other means which have also been found to alter macrophage ability to internalize apoptotic cells are; by activating macrophage signaling pathways (e.g., PKC) [110-112], ligating extracellular matrix receptors [108,113], use of certain cytokines (e.g., IL-10, GM-CSF, TGF- $\beta$ ) [114,115] or eicosanoids (e.g., lipoxins) [116,117]. Recent breakthroughs regarding the ability of glucocorticoids to increase the phagocytic potential of macrophages to remove apoptotic cells by innocuous non-inflammatory means have proved invaluable in aiding the

understanding of their action [7,83,118]. This could be linked to studies revealing that glucocorticoids have the ability to transform the cytoskeletal organization and the protein generating ability of monocytes driving them to become highly phagocytic macrophages [82]. Interestingly, it has been proposed that the cytokine environment present at the site of inflammation may influence the effectiveness of glucocorticoids having implications on their therapeutic use. For example, IFN- $\gamma$  has the ability to counteract the increased phagocytic ability of macrophages obtained from glucocorticoid treated monocytes without altering their morphology [83]. This increase in the phagocytic ability of macrophages through exposure to glucocorticoids complements glucocorticoids capacity to up-regulate eosinophil and lymphocyte apoptosis, possibly explaining why the use of glucocorticoids proves to be a successful treatment for a range of inflammatory diseases.

## THERAPEUTIC APPLICATIONS AND DRUG DISCOVERY

The safe engulfment and removal of apoptotic cells is an emerging target for the treatment of inflammatory diseases. Indeed, this mechanism has now been attributed to many of the drugs that are already in clinical use, such as the glucocorticoids and NSAIDs. Glucocorticoids have been shown to induce apoptosis of inflammatory cells (except neutrophils), and importantly, to promote phagocytosis of these effete cells ensuring their safe removal thus limiting tissue damage and injury. In addition, there are many endogenous 'brake' mechanisms that halt inflammation and actively drive the resolution of inflammation. These include the cyclopentanone prostaglandins, NF- $\kappa$ B p50/50, lipoxins/resolvins, annexin 1 (formerly known as lipocortin 1) and caspases, as previously discussed in this review. These have been shown to induce apoptosis and/or phagocytosis in addition to their other known anti-inflammatory properties [11,50]. Stable analogues and structurally related analogues of cyclopentanone prostaglandins and lipoxins show some promise in various *in vivo* models of inflammation and may prove useful in the treatment of non-resolving inflammatory disorders, such as arthritis, psoriasis and asthma [11]. There are now a number of novel anti-inflammatory strategies under development that target the regulation of apoptosis. In particular there are promising drugs that interfere with caspase activation to influence apoptosis already in phase I and phase II clinical trials for the treatment of rheumatoid arthritis, sepsis, hepatitis and stroke [119-121].

The forced selective induction of apoptosis is thought to be beneficial for the treatment of inflammatory diseases due to a variety of reasons. Primarily, the removal of potentially cytotoxic cells in a safe manner that maintains membrane integrity prevents potential damage to surrounding tissues. Also, phagocytosis of apoptotic cells leads to the generation of anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$ <sub>1</sub> and also may have other unknown endogenous pro-resolving mechanisms that aid resolution of inflammation. Indeed, previous research has shown that the exogenous administration of apoptotic cells induces resolution in an experimental model of inflammation [122]. Interestingly, autologous administration of apoptotic cells has also been clinically proven to prevent heart, kidney and lung transplant rejection [123]



and limit the need for immunosuppressive drugs, which have many associated side effects and weakens the patient's immune system to kill invading pathogens and bacteria. Recent research shows that the engulfment of the apoptotic cells induces tolerogenic changes of immature dendritic cells, which then stimulates inhibitory T regulatory cells to suppress immune rejection [124]. This clearly shows that the administration of apoptotic cells and their safe engulfment in humans has a role in the adaptive and the innate immune system by improving tolerance and promoting the resolution of inflammation.

Autoimmunity may occur due to the failure to clear apoptotic cells resulting in necrosis with the loss of cell membrane integrity, which causes the release of cytotoxic contents that initiates damage to surrounding tissues. This has been shown in approximately half of the patients with systemic lupus erythematosus where failed clearance leads to the prevalence of apoptotic cells, which has been correlated with disease severity [125]. It would be beneficial to assess if the clearance defects of the phagocytic cells could be overcome in order to promote the safe removal of these unwanted cells and what outcome this strategy could have on limiting disease progression or give an improved prognosis for these patients. It is thus becoming clearer that the induction of apoptosis of inflammatory cells could be a beneficial target for the treatment of inflammatory diseases, not only to ensure their safe removal preventing tissue damage but also to elicit an anti-inflammatory cascade of events that promote the resolution of inflammation.

## CONCLUSION

Granulocyte apoptosis is a highly regulated process in that there are many complex interrelated signaling pathways and external stimuli controlling this cell death program. There are many similarities and differences between the control of neutrophil and eosinophil apoptosis. We are beginning to understand the biochemical and molecular checkpoints that control granulocyte apoptosis and it is our belief that they may be exploited for therapeutic gain for the treatment of chronic inflammatory diseases. It would therefore be advantageous to enhance the resolution phase of established inflammation by modifying appropriately apoptosis of specific inflammatory cells and enhancing the clearance of apoptotic cells by phagocytes.

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# Novel pharmacological strategies for driving inflammatory cell apoptosis and enhancing the resolution of inflammation

John M. Hallett<sup>\*</sup>, Andrew E. Leitch<sup>\*</sup>, Nicola A. Riley, Rodger Duffin, Christopher Haslett and Adriano G. Rossi

MRC Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh Medical School, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

**Resolution of inflammation requires the effective downregulation of key inflammatory cells such as neutrophils and eosinophils, which normally undergo programmed cell death (apoptosis) to enable their detection and removal by phagocytes such as macrophages. Dysregulation of this process is thought to contribute to the pathogenesis and progression of chronic inflammatory disorders such as chronic obstructive pulmonary disease, asthma, rheumatoid arthritis, allergic rhinitis and inflammatory bowel disease. Importantly, knowledge of the signalling pathways responsible for the induction and execution of granulocyte apoptosis and the phagocytic removal of apoptotic cells continues to increase and, with it, the potential for incisive pharmacological intervention. In this article, we highlight pharmacological strategies that could be used to drive the resolution of inflammation by augmenting apoptosis of inflammatory cells.**

## Introduction

The inflammatory process is a complex series of inter-related, ideally tightly controlled, cellular and biochemical events that has evolved to eliminate or contain infectious agents and to repair damaged tissue. An ineffective or uncontrolled inflammatory response contributes to the cellular dysfunction, tissue damage and inadequate repair that occurs in many chronic inflammatory diseases. Currently, knowledge of the triggers and progression of the inflammatory process exceeds that of the events responsible for the termination and ultimate resolution of inflammation [1,2].

A central paradigm of inflammation research has been that the resolution of inflammation depends on apoptosis of inflammatory cells (e.g. neutrophils and eosinophils) and their subsequent clearance by phagocytes (especially macrophages) [3]. This process is synchronized by an inter-connecting web of signalling pathways that not only down-regulates the inflammatory response but also generates agents capable of driving its resolution. In this review, we

focus on the granulocyte, which we consider to be the archetypal inflammatory cell, and discuss the pharmacological interventions that have stemmed from a greater understanding of its role [4].

## *The granulocyte life cycle*

Granulocytes – the collective name given to neutrophil, eosinophil and basophil leukocytes – have a prominent role in immune defence [5]. As the most abundant circulating granulocyte in human blood, neutrophils have a key role in the defence against bacterial, fungal and viral infections. Eosinophils account for <5% of the circulating granulocytes and confer resistance to parasitic invasion, in addition to their involvement, with basophils (the third, and numerically scarce, type of granulocyte), in the allergic response [6].

Granulocytes are derived from pluripotent haematopoietic stem cells in the bone marrow. If a cell is destined to become a granulocyte, it must differentiate from a common myeloid progenitor cell to become a common granulocyte progenitor cell, which, following appropriate stimulation, can produce any granulocyte lineage. Granulocytes are terminally differentiated and, having effectively completed the cell cycle, they remain in the G0 phase of growth for the remainder of their relatively short lives [7]. It is believed that, once they have discharged their function, extravasated granulocytes in the tissues die by apoptosis (see next section), whereas non-migratory, circulating granulocytes leave the blood and return to the liver, spleen or bone marrow, where they meet an ill-defined fate (possibly apoptosis) [8]. The life cycle of the granulocyte and its differentiation in particular are under increasing scrutiny. A new pharmacological intervention – cyclin-dependent kinase inhibition – targets the cell-cycle machinery and drives granulocyte apoptosis [9].

## *Granulocyte apoptosis*

Apoptosis is a complex physiological mechanism in which a cell undergoes programmed death as a result of survival-factor withdrawal or exposure to pro-apoptotic signals (Box 1). The alternative to granulocyte apoptosis (Figure 1) is necrosis, which can be a primary event or which can

Corresponding author: Rossi, A.G. (a.g.rossi@ed.ac.uk).

<sup>\*</sup> Authors contributed equally.

### Box 1. Apoptotic pathways and the role of caspases

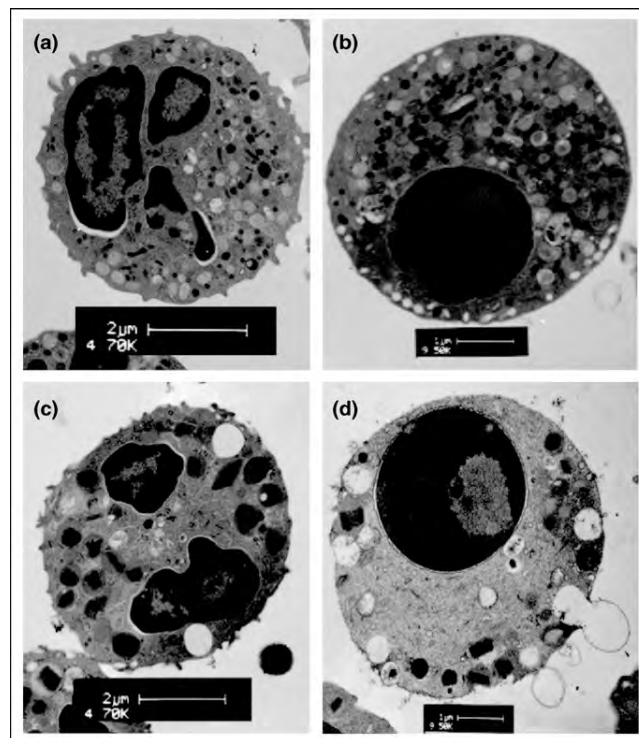
#### The extrinsic pathway

The extrinsic apoptotic pathway is initiated by stimulation of the TNF receptor (TNFR) superfamily of cell-surface 'death receptors', which includes TNF-related apoptosis-inducing ligand receptor, TNFR and Fas. On binding to TNFR1, TNF- $\alpha$  induces receptor trimerization, which co-localizes death domains and enables further binding to TNFR-associated death domain-containing proteins (TRADDs). TRADD mediates clustering of procaspase-8 molecules and their proximity to one another initiates an autocatalytic activation cascade leading to apoptosis. (Similarly, Fas-associated death domain protein, FADD, mediates the same reaction following Fas-receptor ligation.) Interestingly, via binding through TNFR-associated factor-2 and receptor-interacting protein, TRADD can activate the transcription factors AP-1 and NF- $\kappa$ B, with the latter having a pivotal role in the immune response. Caspase-8 can either directly cleave procaspase-3 to caspase-3 (the terminal step in the cascade preceding cleavage of DNA and chromatin) or—in an example of cross-talk between extrinsic and intrinsic pathways—truncate the Bcl-2 family member Bid: a step that contributes to MOMP [73,74]. CD137 is another member of the TNFR family (despite lacking a death domain), which seems to have varying effects on eosinophil and neutrophil survival signalling [65].

#### The intrinsic pathway

MOMP, which is mediated by a predominance of pro-apoptotic over anti-apoptotic Bcl-2 family members, is the central event in the intrinsic pathway, which is stimulated by genotoxic stress (e.g. cytotoxic agents, UV radiation or oxidative stress). Cytochrome *c*, apoptotic-protease-activating factor-1, apoptosis-inducing factor, endonuclease G and Smac/DIABLO are released following MOMP. Smac/DIABLO is required for the release of procaspase-9 from IAPs, enabling formation of the apoptosome and activation of caspase-3, which initiates apoptosis. It has been suggested that, whereas caspase-9 is essential for neutrophil and eosinophil apoptosis, caspase-3 and caspase-8 could have less significance in eosinophil apoptosis – a difference that was hoped might have potential for exploitation. It should be noted, however, that the importance of caspases to constitutive apoptosis has not yet been satisfactorily confirmed [21,66–68].

supersede apoptosis when phagocytosis is delayed. Apoptosis, in contrast to necrosis, ensures that the cell membrane retains its integrity, preventing spillage of the inherently pro-inflammatory and, especially in the case of neutrophils, histotoxic contents of the cell into the surrounding tissues. The release of histotoxic products [e.g. proteases or reactive oxygen species (ROS)] from over-recruited, inappropriately activated and/or necrotic granulocytes probably contributes to the tissue damage observed in chronic inflammatory and autoimmune diseases [2–4,10]. However, there is growing evidence that potentially harmful neutrophil granule contents such as ROS or cathepsin G have important pro-apoptotic roles in the successful resolution of inflammation [10,11]. Another important component of granulocyte apoptosis is the preparation of the cell for recognition and removal by phagocytes. During apoptosis, receptors for pro-inflammatory signals are removed from the cell membrane, whereas 'eat me' signals [including phosphatidylserine (PS) residues], which are recognized by phagocytes, are displayed. A standard tool for the identification of apoptotic cells involves annexin-V binding to PS residues. Interestingly, a putative phagocyte receptor for PS has proved difficult to define, and three recently identified receptors (Tim4, stabilin2 and BAI1) indicate that the complexity of this



**Figure 1.** Electron micrographs of granulocytes. (a) A healthy neutrophil, identifiable by its three-lobed nuclei and heavily granular structure (with a 2- $\mu$ m scale bar taken at 4700 $\times$  magnification). (b) An apoptotic neutrophil with dense chromatin condensation and smooth cell membrane (with a 1- $\mu$ m scale bar taken at 9500 $\times$  magnification). (c) A healthy eosinophil with two-lobed nuclei, large granules and Charcot-Leyden crystals (crystals comprising eosinophil lysophospholipase: a hallmark of eosinophilic infiltration) (with a 2- $\mu$ m scale bar taken at 4700 $\times$  magnification). (d) An apoptotic eosinophil showing chromatin condensation, peripheral granular distribution, smooth cell membrane and vacuolated appearance (with a 1- $\mu$ m scale bar taken at 9500 $\times$  magnification).

process might have been underestimated [12–16]. Phagocytosis should be non-phlogistic, implying that a professional phagocyte such as the macrophage – which, in other circumstances, can propagate inflammation – on recognizing an apoptotic cell, is programmed to upregulate the production of anti-inflammatory mediators such as transforming growth factor (TGF)- $\beta$  and interleukin (IL)-10 [17]. Developing an understanding of granulocyte behaviour has been a research priority, and has indicated that a therapeutically tractable approach to the modulation of granulocyte functional responsiveness might be to influence the longevity of these cells by inducing apoptosis [18]. Additionally, research is generating an increasingly detailed picture of the apoptotic machinery at a molecular level. This information is crucial for the accurate identification of apoptotic cells and could provide novel opportunities for intervention.

### Key signalling pathways as targets for pharmacological intervention

Granulocyte apoptosis is a sensitive, responsive and highly regulated process. These features are conferred by various signalling pathways. Each pathway is responsive to specific endogenous stimuli and mediates pro- or anti-apoptotic effects. As such, a key area for pharmacological intervention is the inhibition or augmentation of key granulocyte signalling pathways (Table 1).



**Table 1. Current pharmacological agents proven to drive granulocyte apoptosis or resolution of inflammation**

Drug	Action	Outcome	Influence on granulocyte apoptosis	Resolution of inflammation	Refs
Glutathione	NF- $\kappa$ B inhibition	Decreased NF- $\kappa$ B activation	Yes	Possible, but probably nonspecific toxicity	[20]
MG132	Proteasome inhibition	Increased Mcl-1, decreased NF- $\kappa$ B activation	No	No	[36,69]
PGD <sub>2</sub> metabolites	Block I $\kappa$ B degradation	Decreased NF- $\kappa$ B activation	Yes	Yes	[22,70–72]
PD098059	Decreased ERK phosphorylation	ERK inhibition	Yes	Yes	[31,32]
SB203580	p38 MAPK inhibition	p38 MAPK inhibition	No	No	[33–36]
Sodium salicylate	p38 MAPK activation	Decreased Mcl-1	Yes	Yes	[36]
SP600125	JNK inhibition	TNF- $\alpha$ -mediated apoptosis	Yes	Yes	[37–39]
Wortmannin	PI3K inhibition	Decreased Akt phosphorylation and IL-5	Possible	Possible, but probably nonspecific toxicity	[44]
Ly294002	PI3K inhibition	Decreased Akt phosphorylation and IL-5	Possible	Possible, but probably nonspecific toxicity	[44]
IC87114	PI3K- $\delta$ inhibition	Decreased chemotaxis	No	Possible	[45]
AS605240	PI3K- $\gamma$ inhibition	Decreased chemotaxis	No	Possible	[45]
CDKi	?	Decreased Mcl-1-X-IAP	Yes	Yes	[9,58–62]
Smac-DIABLO mimetics	Decreased X-IAP	Increased ease of apoptosis	Unknown	Unknown	[51,52]
Glucocorticoids	Decreased Mcl-1 in eosinophils	Eosinophil apoptosis	Eosinophil apoptosis only	Eosinophilic inflammation	[1,3,49]

### NF- $\kappa$ B: a crucial regulator of granulocyte apoptosis

The transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) has a pivotal role in the inflammatory response, and a plethora of molecules – including cytokines, chemokines, adhesion molecules and stress response proteins – is dependent on it for synthesis. The synthesis of several proteins under the direct transcriptional control of NF- $\kappa$ B are anti-apoptotic in nature, including B-cell lymphoma-2 [Bcl-2 (which is less relevant in neutrophils, in which it is not expressed)], inhibitor of apoptosis proteins (IAPs) and X-ray-inducible immediate-early-response factor-1-long [19]. Specific inhibition of NF- $\kappa$ B is an attractive pharmacological target that is made viable by the ever-increasing understanding of its activity and regulation.

Inactive NF- $\kappa$ B is held in the cytoplasm bound to its inhibitor protein, inhibitor of  $\kappa$ B (I $\kappa$ B). However, once activated by pro-inflammatory stimuli such as tumour necrosis factor (TNF)- $\alpha$  or lipopolysaccharide (LPS), the I $\kappa$ B kinase (IKK) complex phosphorylates I $\kappa$ B. I $\kappa$ B then dissociates from NF- $\kappa$ B to be ubiquitinated and targeted for proteasomal degradation. As a result of I $\kappa$ B degradation, nuclear-localization sequences (NLSs) on NF- $\kappa$ B are revealed and NF- $\kappa$ B subunits are free to translocate to the nucleus and begin the process of protein transcription. From a pharmacological perspective, an obvious intervention would be to augment I $\kappa$ B suppression of NF- $\kappa$ B; however, this would be difficult because I $\kappa$ B is rapidly biodegraded [18,19].

Several pharmacological inhibitors of NF- $\kappa$ B are available and have been shown to enhance apoptosis when applied to neutrophils *in vitro*. These inhibitors include cell-permeable inhibitory peptide of NF- $\kappa$ B, SN-50, curcumin, pyrrolidine-dithiocarbamate and MG132 (a proteasome inhibitor the impact of which could be considered nonspecific). A widely studied inhibitor is the fungal metabolite gliotoxin, which induces neutrophil apoptosis *in vitro* and can overcome the neutrophil survival effect conferred by LPS. The effects of TNF- $\alpha$  on granulocyte apoptosis are perplexing, although it has been shown that, in combination with gliotoxin, it induces marked neutrophil and eosinophil

apoptosis [20]. In addition, cycloheximide-mediated inhibition of protein synthesis augments the effect of TNF- $\alpha$ , implying the presence of a TNF- $\alpha$ -induced survival protein that is under the transcriptional control of NF- $\kappa$ B. In the search for physiological inhibitors of NF- $\kappa$ B, it was noted that arachidonic acid metabolites and the cyclopentenone prostaglandin (PG)D<sub>2</sub> could inhibit NF- $\kappa$ B activation by LPS or TNF- $\alpha$  and induce caspase-dependent granulocyte apoptosis. This effect was independent of the peroxisome-proliferator-activated-receptor- $\gamma$  and probably occurred by direct IKK inhibition [19,21]. There is now *in vivo* evidence, from a rat pleurisy model, that PGD<sub>2</sub> can contribute to the resolution of inflammation and could be a natural retardant of inflammation progression [22].

An innovative and encouraging use of the HIV tat protein transduction method has facilitated the transduction of neutrophils with HIV-tat-linked NF- $\kappa$ B essential modulator domain (NEMO), which is a specific inhibitor of the IKK $\gamma$ -IKK $\beta$  interaction [23]. A similar approach was used to transduce eosinophils with an HIV-tat-I $\kappa$ B $\alpha$  super-repressor that is resistant to phosphorylation and proteasomal degradation [24]. This highly specific inhibition resulted in the augmentation of apoptosis and the suppression of NF- $\kappa$ B-mediated survival, lending credence to the pursuit of NF- $\kappa$ B as a pharmacological target.

However, an apparent contradiction is emerging in the literature because NF- $\kappa$ B has been shown to have an anti-inflammatory and pro-resolution role in acute inflammation. The contradiction revolves around detailed analysis of IKK $\alpha$  inhibition of NF- $\kappa$ B activity, in which induction of the NF- $\kappa$ B subunits RelA and c-Rel using a super-repressor I $\kappa$ B $\alpha$  fused to a membrane-transducing domain of HIV tat protein suppressed NF- $\kappa$ B interaction with pro-inflammatory gene promoters *in vivo*. It is now recognized that there are two phases of NF- $\kappa$ B activation. The first phase, which occurs within 6 h of the onset of inflammation, is coincident with the formation of complexed inducible nitric oxide synthase (iNOS) and cRel-p50 heterodimer; these bind to and regulate the mouse iNOS promoter, and macrophages from knockout mice

show increased iNOS expression in response to NF- $\kappa$ B during the induction of inflammation *in vivo*. During the resolution phase, however, there is no expression of iNOS or cRel-p50 heterodimers despite substantial NF- $\kappa$ B activation, which indicates a switch to a second, pro-resolution phase [25–27].

It seems that the dominant impact of NF- $\kappa$ B inhibition is to drive granulocyte apoptosis and the resolution of inflammation, but a note of caution is warranted because it is conceivable that inhibition of the later, pro-resolution phase of NF- $\kappa$ B activation might prolong and/or prevent the successful resolution of inflammation.

#### *The role of MAPK pathways in granulocyte apoptosis*

There is considerable evidence of the involvement of the mitogen-activated protein kinase (MAPK) signalling cascade in diverse aspects of the inflammatory response, including cell recruitment, activation and apoptosis. The three main MAPK subtypes activated by MAPK kinases (MEKs) 1–7 are extracellular-signal-related kinase (ERK (which includes p42/p44 MAPK)), c-Jun N-terminal kinases (JNKs) and p38 MAPK. All have been implicated in the regulation of granulocyte apoptosis [28–30].

The ERK pathway is involved in negotiating survival conferred by inflammatory mediators such as granulocyte macrophage-colony stimulating factor (GM-CSF), IL-8, IL-15, C5a and LPS but it is non-essential for the regulation of constitutive apoptosis. PD098059 (2'-amino-3'-methoxyflavone) is a specific pharmacological ERK inhibitor that mediates its effect by binding to the ERK-specific MAP-kinase, thereby preventing the phosphorylation of ERK1/2 (p44/p42 MAPK) by MEK1. PD098059 has been used *in vitro* to reverse GM-CSF-mediated and LPS-mediated neutrophil survival, and its *in vivo* efficacy has been demonstrated in a rat carrageenan-induced pleurisy model, in which it enhanced the resolution of inflammation by promoting neutrophil apoptosis within the pleural cavity. It is likely that the pro-apoptotic effects of this compound are conferred by inhibition of survival-factor-induced anti-apoptotic effects because it seems to have no direct effect on constitutive neutrophil apoptosis in non-inflammatory conditions [31,32].

The p38 MAPK and JNK cascades are involved in the cellular response to stresses, including heat shock, hyperosmolarity, UV radiation, protein-synthesis inhibitor drugs and inflammatory cytokines. p38 MAPK might also have a role in superoxide anion production and chemotaxis [33]. There is indirect evidence of a pro-survival role for p38 MAPK because it has been shown to inhibit, by phosphorylation, caspase-3 and -8 and it is specifically cleaved in mature neutrophils undergoing apoptosis [34]. However, it is unclear whether it is sufficiently influential to be considered a pharmacological target in inflammatory states because its inhibition (by the pyridinyl imidazole inhibitor SB203580) fails to overcome cell-survival signals from GM-CSF. It has been suggested that this controversial pathway could generate a neutrophil death signal through the reduction of Mcl-1, as observed when apoptosis is triggered by sodium salicylate. Perhaps there are further opportunities for the pharmacological augmentation of p38 MAPK to promote neutrophil apoptosis. There is certainly

an opportunity for further study of this pathway, which seems to have been under-investigated compared with other signalling systems [35,36].

The JNK pathway is also controversial and insufficiently investigated, but researchers have demonstrated that the activation of JNK signalling is a component of LPS- and TNF- $\alpha$ -mediated neutrophil apoptosis. Additionally, an *in vivo* rat model of smoke inhalation injury responded favourably to JNK inhibition by SP600125 (anthra [1,9-cd]pyrazol-6(2H)-one), with decreased airway inflammation and increased rates of survival [37–39].

#### *PI3K pathway as a regulator of granulocyte apoptosis*

The phosphoinositide-3-kinase (PI3K) pathway is an important cell-surface-receptor-controlled signal-transduction pathway that catalyses phosphorylation of the 3'-OH position of the inositol ring of phosphoinositides, resulting in the formation of the 3'-phosphatidylinositol (PtdIns) lipids. PI3K is involved in the regulation of all major leukocyte events, including growth, proliferation, recruitment, activation and survival [40]. From the perspective of granulocyte survival, PI3K has a role in mediating the anti-apoptotic-pro-inflammatory signals triggered by LPS, GM-CSF and TNF- $\alpha$ . It generates the important signalling lipid PtdIns(3,4,5) $P_3$ , which activates Akt and influences NF- $\kappa$ B and cAMP-response-element-binding protein (CREB); these produce anti-apoptotic signals in granulocytes [41–43].

Pharmacological inhibition was initially achieved with the use of wortmannin and LY294002, which are general PI3K family inhibitors. Work is in progress to determine which isoforms of PI3K are responsible for its diversity of function and to delineate their mechanisms of action. To this end, PI3K-isoform-specific knockout mice have been developed alongside new pharmacological inhibitors. For example, the involvement of PI3K in the recruitment and survival of eosinophils in a model of allergic pleurisy in PI3K $\gamma$ -deficient mice with the use of wortmannin and LY294002 has been evaluated [44]. They found that, following the induction of allergic pleurisy, eosinophil accumulation was not different from that in wild-type mice at earlier time-points (6 and 24 h). However, there was a marked reduction in eosinophil numbers at a later time-point (48 h). Wortmannin and LY294002 administered systematically before antigen challenge led to a decrease in Akt phosphorylation, IL-5 production and eosinophil release from the bone marrow and, consequently, prevented the recruitment of eosinophils. Importantly, local (intrapleural) treatment with the PI3K inhibitors 24 h after antigen challenge (at the peak of inflammation) enhanced the clearance of accumulated eosinophils – an effect associated with the inhibition of Akt phosphorylation and an increased number of apoptotic events (assessed microscopically and using annexin-V binding). This study highlights an important role for the PI3K family in the accumulation and survival of eosinophils and implies a specific role in the maintenance (possibly by influencing eosinophil apoptosis) of eosinophilic inflammation *in vivo*. The same group has now used the specific PI3K- $\delta$  inhibitor IC87114 and the PI3K- $\gamma$  inhibitor AS605240 to demonstrate that, whereas chemokine receptor (CXCR)2 requires

both isoforms to recruit neutrophils to the alveolar space, C5a and formyl-methionyl-leucyl-phenylalanine (fMLP) require only PI3K $\gamma$  [45].

Pharmacological inhibitors of specific PI3K isoforms and the development of knockout mice have demonstrated the importance of this signalling pathway to the granulocyte and the wider inflammatory response.

#### The Bcl-2 family

The Bcl-2 family, which contains >20 members, comprises a series of cell-death-receptor proteins. These are further classified into three subgroups depending on their function and the number of Bcl-2 homology domains that they contain: the multidomain pro-apoptotic members, the Bcl-2 homology 3 (BH3)-only domain pro-apoptotic members and the anti-apoptotic family. Neutrophils and eosinophils express the multidomain pro-apoptotic family members Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak), and the BH3-only domain pro-apoptotic members BH3-interacting domain death agonist (Bid) and Bcl-2-associated death promoter (Bad), which are essential components of the apoptotic machinery. Neutrophils also express the pro-apoptotic BH3-only domain proteins Bim and p53 upregulated modulator of apoptosis (PUMA). These pro-apoptotic Bcl-2 family proteins are thought to function either by translocation to the mitochondria, where they can influence mitochondrial outer-membrane permeabilization (MOMP), or by binding to and downregulating pro-survival proteins. In fact, the role of mitochondria in neutrophils and eosinophils seems to be geared predominantly towards the induction of apoptosis rather than cellular respiration [46].

Anti-apoptotic members of the Bcl-2 family are also present in granulocytes. Neutrophils express the Bcl-2 homologue A1 and the anti-apoptotic proteins myeloid cell leukaemia-1 (Mcl-1), Bcl-X<sub>L</sub> and X-linked mammalian IAP (X-IAP). Survival signals, in the form of cytokines and other mediators found at sites of inflammation, induce *de novo* synthesis of these anti-apoptotic proteins [47,48].

Mcl-1 was initially isolated from a human myeloblastic leukaemia cell line and it is increasingly recognized as a key regulator of immune-cell proliferation and longevity. In human neutrophils, Mcl-1 is thought to be important for cytokine-regulated survival but does not seem to be transcribed by NF- $\kappa$ B, which (as discussed) is one of the most important inflammatory transcription factors. Levels of Mcl-1 in human neutrophils correlate closely with neutrophil survival kinetics. Mcl-1 levels are elevated in viable cells and decrease before the induction of apoptosis. This protein also has the fastest turnover rate and, therefore, the shortest half-life of the anti-apoptotic members of the Bcl-2 family: a feature that is compatible with the necessarily short half-life of the neutrophil. Mcl-1 is targeted for degradation in the proteasome following ubiquitylation and undergoes significant post-translational modification, providing further opportunities for pharmacological intervention. Glucocorticoids are well-established anti-inflammatory agents that promote eosinophil apoptosis but extend neutrophil longevity. They are important in the management of eosinophil-dominant diseases such as asthma but are less effective in neutrophil-dominant

diseases such as chronic obstructive pulmonary disease. Partial efficacy in neutrophil-dominant disease is probably achieved by effects on the downregulation of inflammatory cytokines and the enhancement of macrophage phagocytosis of apoptotic neutrophils [75]. The glucocorticoid dexamethasone has recently been shown to stabilize Mcl-1 levels in neutrophils but to reduce levels in eosinophils, which might account for this dichotomous effect on granulocytes [50]. An important development in Mcl-1 research has been the advent of a myeloid-specific Mcl-1-knockout mouse, which has a normal, functional macrophage population but which lacks neutrophils – demonstrating the pivotal survival role of Mcl-1 in neutrophils [51]. A putative inhibitor should induce neutrophil apoptosis but enable the effective clearance of apoptotic cells by macrophages. As such, Mcl-1 could be a key pharmacological target. Our experience with the cyclin-dependent kinase (CDK) inhibitor R-roscovitine indicates that it adequately fulfils the criteria [9].

Members of the IAP family of proteins, of which X-IAP seems to be the most potent, inhibit both caspase-9 (an initiator caspase) and the effector caspases-3 and -7, thereby promoting cell survival. In addition, overexpression of X-IAP protects cells from apoptosis (see Refs [51–54]). X-IAP is negatively regulated by X-IAP-associated factor 1, which is found predominantly in the nucleus, and by Smac/DIABLO (second-mitochondria-derived-activator of caspase/direct inhibitor of apoptosis-binding protein with low pI), which is located in the mitochondria [52,53]. Using NMR and X-ray crystallography, recent characterization of the Smac–IAP interaction has enabled the design and testing of Smac/DIABLO mimetics that have *in vitro* and *in vivo* activity [52,53]. The implications for granulocyte apoptosis have not yet been established. Interestingly, stabilization of X-IAP by the ERK pathway has been described, and this effect could be reversed by oxidant-induced p38 MAPK [54]. This provides an important insight into the signalling pathway cross-talk that is present at inflammatory loci [54].

Bcl-X<sub>L</sub> is a Bcl-2 homologue that conserves all four BH domains. Experiments using *in vivo* rat carrageenan-induced pleurisy models have shown that, during the onset of inflammation, increased expression of Bcl-X<sub>L</sub> is accompanied by significantly decreased levels of Bax expression. Conversely, during resolution of inflammation, Bax expression was increased and negligible levels of Bcl-X<sub>L</sub> were found. In the same experiment, the inhibition of Bax by V5 (a Bax inhibitory peptide) resulted in prolonged inflammation, supporting the hypothesis that Bax has a crucial role in the induction of neutrophil apoptosis, and thereby facilitates the resolution of inflammation [31]. There is clearly a delicate balance between the influence of pro-survival and anti-apoptotic proteins within the granulocyte, whereby processes that mediate the effective clearance of inflammatory stimuli compete with those that result in a counter-productive prolongation of inflammation.

#### Lipoxins, resolvins and protectins: pro-resolution lipid mediators

Polyunsaturated fatty acids can be metabolized to arachidonic acid, eicosapentaenoic acid and docosahexaenoic



acid, which can be processed to lipoxins, resolvins and protectins, respectively. These lipid mediators are increasingly recognized for their potent anti-inflammatory and pro-resolution roles and might have a modulatory role in several inflammatory conditions such as atherosclerosis, chronic liver disease, inflammatory bowel disease, glomerulonephritis and asthma [1]. Lipoxins have diverse actions, including the inhibition of neutrophil chemotaxis, adhesion and transmigration. Additionally, they mediate normalization of vascular permeability, non-phlogistic recruitment of mononuclear cells and stimulation of macrophage phagocytosis of apoptotic neutrophils. Aspirin-triggered lipoxins seem to target the same seven-transmembrane G-protein-coupled receptor as does glucocorticoid-induced annexin-1. Annexin-1 induces granulocyte apoptosis but is also released by both neutrophils and macrophages to enhance the phagocytosis of apoptotic cells. This is interesting because it implies that lipoxins might participate in an interconnected anti-inflammatory network and pro-resolution cascade [55,56]. Resolvins and protectins share some of the actions of lipoxins and have protective roles in many organ systems. Importantly, the resolution of inflammation has been reported *in vitro* and in a mouse peritonitis model with resolvin E1 and protectin D1 [57]. The anti-inflammatory and pro-resolution effects of this family of mediators have been demonstrated *in vitro* and, latterly, *in vivo*, indicating that it will be an important target for novel anti-inflammatory pharmacotherapeutics [49].

#### CDK inhibition as a novel anti-inflammatory approach

CDK inhibitors have been used for the selective induction of apoptosis in actively proliferating cells for several years. Several CDK inhibitors, including R-roscovitine, flavopiridol and SU9516, can induce apoptosis in cancer cell lines. R-roscovitine alone reduces the proliferation index of 19 distinct cancer cell lines *in vitro* [58]. The induction of apoptosis by CDK inhibitors seems to be mediated by the modulation of Bcl-2 family members and to be executed in a caspase-dependent manner. A wealth of research has meant that confidence in CDK inhibitor safety and efficacy has increased to the extent that clinical trials are underway for conditions such as B-cell malignancy, non-small-cell lung cancer and breast cancer. So far, the side-effect profile of CDK inhibitors has been promising but it includes tolerable gastrointestinal disturbance, minor hepatic dysfunction and hypokalaemia [59–61].

Logically, in terminally differentiated cells such as granulocytes, CDK inhibitors should have no effect because the cell-cycle machinery ought to be redundant. In fact, in post-proliferation neurons, CDK inhibitors have been shown to have a protective effect against apoptosis [62]. Nonetheless, we recently demonstrated *in vitro* that a panel of CDK inhibitors induces neutrophil apoptosis in a time- and concentration-dependent manner and that this effect is sufficient to overcome the anti-apoptotic effects of survival factors such as GM-CSF, dibutyryl-cAMP and LPS [9]. When neutrophils were treated with R-roscovitine and the caspase inhibitor zVAD-fmk (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) cells failed to enter apoptosis, indicating that R-roscovitine functions in a

caspase-dependent manner. Furthermore, *in vitro* studies have demonstrated the presence of CDK1 and CDK2 in human neutrophils and have shown that levels of both remain constant throughout ageing, treatment with GM-CSF or gliotoxin, which indicates that these kinases are not targeted for degradation during apoptosis [9]. In addition, a reduction in Mcl-1 levels after treatment of neutrophils with R-roscovitine was noted, offering a tantalizing insight into its mechanism of action [9]. The same study [9] assessed the impact of R-roscovitine on models of neutrophil-dominant inflammation, including carrageenan-induced pleurisy, bleomycin lung injury and arthritis in mice. The results indicated a resolution of inflammation – as assessed by several parameters, including cytokine levels, inflammatory cell numbers, histology and arthritis scores. Caspase inhibition *in vivo* with zVAD-fmk, which reversed the pro-resolution effect, confirmed the integral role of granulocyte apoptosis in resolution. In addition, recent studies have identified a role for CDK5 in neutrophils, and CDK inhibition has been shown to influence inflammatory cell recruitment [63,64]. Further elucidation of the mechanism of action of CDK inhibitors could enable this therapy to be refined and might highlight further targets for pharmacological intervention.

#### Concluding remarks

Research into granulocyte apoptosis and its role in the resolution of inflammation has revealed a complex web of signal-transduction pathways. Work has begun on the pharmacological manipulation of these pathways to provide novel treatments for inflammatory disorders. Clearly, the rate of neutrophil or eosinophil apoptosis can be increased or reduced *in vitro* by several agents. Importantly, this rate can be matched by appropriate clearance of apoptotic cells. There is now conclusive evidence that inhibition or promotion of granulocyte apoptosis by pharmacological agents *in vivo* can delay or enhance the resolution of acute inflammation, respectively. Ways to limit the toxicity of some of these agents must be found but we can be confident of the transition of others, in the near future, to clinical trials.

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# The cyclin-dependent kinase inhibitor R-roscovitine down-regulates Mcl-1 to override pro-inflammatory signalling and drive neutrophil apoptosis

Andrew E. Leitch, Nicola A. Riley, Tara A. Sheldrake, Michela Festa, Sarah Fox, Rodger Duffin, Christopher Haslett and Adriano G. Rossi

MRC Centre for Inflammation Research, The Queen's Medical Research Institute, University of Edinburgh Medical School, Edinburgh, Scotland, UK

Successful resolution of inflammation requires inflammatory cells such as neutrophils to undergo apoptosis prior to non-inflammatory phagocytosis by professional phagocytes. Recently, cyclin-dependent kinase (CDK) inhibitors (e.g. R-roscovitine) have been shown to induce neutrophil apoptosis and enhance the resolution of inflammation. Interestingly, NF- $\kappa$ B and MAPK pathways and key endogenous survival proteins (typified by Mcl-1) are involved in the regulation of neutrophil apoptosis and, in cancer-cell lines, have been implicated as possible targets of CDK inhibitors. Here, we demonstrate that R-roscovitine over-rides TNF- $\alpha$  and LPS-induced survival (determined by morphological examination and binding of fluorescently labelled annexin-V) of isolated peripheral blood neutrophils. This effect did not appear to be mediated via effects on early markers of neutrophil activation (e.g. surface marker expression, shape change, aggregation and superoxide anion generation), by direct inhibition of NF- $\kappa$ B activation (assessed by cytoplasmic I $\kappa$ B $\alpha$  proteolysis and NF- $\kappa$ B p65 subunit translocation) and ERK activation (determined by specific ERK phosphorylation) but due to down-regulation (at protein and mRNA level) of the survival protein Mcl-1 but not the pro-apoptotic bcl-2 homologue Bim. These findings suggest that key endogenous survival proteins may be the targets of CDK inhibitors and consequently may be of critical importance in the resolution of inflammation.

**Key words:** Apoptosis • Cyclin-dependent kinase inhibition • Inflammation • Neutrophil

## Introduction

It is essential for the timely resolution of inflammation that neutrophils undergo apoptosis and are effectively cleared by phagocytes [1]. At sites of inflammation, augmented neutrophil survival is conferred by cytokines (e.g. IL-6, IL-8, TNF- $\alpha$  and GM-CSF), bacterial products (e.g. LPS) and local factors such as oxygen tension [2]. These signals greatly extend neutrophil lifespan and facilitate the appropriate up-regulation of neutrophil response to an inflammatory challenge. If this up-regulation is

sustained and becomes persistent it can lead to chronic inflammatory or autoimmune pathology [3, 4]. In the development of anti-inflammatory pharmacological agents that drive neutrophil apoptosis, it is essential to consider the powerfully pro-survival inflammatory milieu that must be overcome [2, 5].

By elucidating the processes responsible for the regulation of neutrophil survival it should be possible to harness them with the aim of driving resolution of inflammation [6]. Activation of the archetypal inflammatory transcription factor NF- $\kappa$ B has been shown to prolong neutrophil lifespan [7]. NF- $\kappa$ B is usually bound and held within the cytoplasm by its physiological inhibitor I $\kappa$ B $\alpha$  and appropriate pro-inflammatory signalling can promote degradation of I $\kappa$ B $\alpha$  and allow NF- $\kappa$ B to translocate to the nucleus where it binds DNA and initiates transcription. NF- $\kappa$ B is

Correspondence: Dr. Andrew Leitch  
e-mail: aleitch3@staffmail.ed.ac.uk

responsible for the transcription of the neutrophil survival protein XIAP, which inhibits caspases 3, 7 and 9 [8]. It has recently been suggested that XIAP may be the critical discriminator between type I (e.g. lymphocytes) and II (e.g. granulocytes) apoptosis [9]. It has also been implicated in neutrophil survival mediated by G-CSF [10] and is degraded in oxidant-induced neutrophil apoptosis [11]. Inhibitors of NF- $\kappa$ B promote neutrophil apoptosis while pro-inflammatory signals such as LPS and TNF- $\alpha$  activate NF- $\kappa$ B to enhance survival [7].

The ERK pathway is involved in negotiating survival conferred by inflammatory mediators such as GM-CSF, IL-8, IL-15, C5a and LPS, but appears to be non-essential for regulation of constitutive apoptosis. PD098059 (2'-amino-3'-methoxyflavone) is a specific pharmacological ERK-inhibitor and mediates its effect by binding to the ERK-specific MAP-kinase MEK, thus preventing phosphorylation of ERK1/2 (p44/p42 MAPK) by MEK1. It has been used *in vitro* to reverse GM-CSF and LPS-mediated neutrophil survival [12]. *In vivo* efficacy of PD098059 has been demonstrated in a rat carrageenan-induced pleurisy model where it enhanced resolution of inflammation by promoting neutrophil apoptosis within the pleural cavity. It is likely that pro-apoptotic effects of this compound are conferred by inhibition of survival factor-induced anti-apoptotic effects as it seems to have no direct effect on constitutive neutrophil apoptosis in non-inflammatory conditions [13, 14].

Neutrophils are the front-line of the innate immune response and therefore possess an array of functions that come to the fore when they become activated by microbes or microbial products. Some of these activation parameters have down-stream consequences in terms of intracellular signaling and cell viability. For example, production of reactive oxygen species typified by the superoxide anion can promote apoptosis [15] while neutrophil activation, typified by shape change and increased adhesiveness, can promote longevity [16]. In assessing a new compound that drives neutrophil apoptosis it is therefore essential to consider these parameters.

The inflammatory response is renowned for multiple layers of complex, redundant regulation and it is therefore not surprising that an important neutrophil survival protein, Mcl-1, is not under the direct transcriptional control of NF- $\kappa$ B. Mcl-1 is a Bcl-2 homologue that promotes neutrophil survival by maintenance of the outer mitochondrial membrane against pro-apoptotic Bcl-2 family members. It is a rapidly transcribed, short-lived protein that is subject to prompt turnover by the proteasome [17]. Mcl-1 transcription and stability can be influenced by various signalling pathways (PI3K, ERK, glycogen synthase kinase, RAF), cytokines (GM-CSF, TNF- $\alpha$ ) and pharmacological agents (dexamethasone, sodium salicylate) [18–21]. It is this sensitivity to the cell's environment and facility for rapid up or down-regulation that place Mcl-1 in the vanguard of apoptotic decision-making.

By contrast, the pro-apoptotic Bcl-2 homologues such as Bim, Bax, Bid and Bad have much longer half-lives and it has usually been assumed that they are a constant but passive presence held in check by anti-apoptotic Bcl-2 homologues. The

active process has been considered to be the depletion of anti-apoptotic proteins allowing their pro-apoptotic counterparts to become effectors almost by default. Recently, it has been demonstrated that Bim plays an important role in the induction of apoptosis in neutrophils stimulated towards enhanced longevity by cytokines such as GM-CSF and IL-3 [22]. Bim was shown to be actively transcribed in the presence of pro-survival cytokines, a finding that was ascribed to a novel function as a physiological brake on cytokine-mediated enhanced neutrophil longevity.

R-roscovitine is a CDK inhibitor that has recently been found to have powerful anti-inflammatory properties [23]. Importantly, R-roscovitine is capable of overcoming survival signals such as GM-CSF to promote neutrophil apoptosis *in vitro* and resolution of inflammation in established animal models. R-roscovitine has been investigated previously from an anti-cancer perspective and has been found to promote apoptosis in myeloma cell-lines by down-regulation of Mcl-1 [24]; indeed R-roscovitine and other CDK inhibitors are undergoing Phase I and II clinical trials for the treatment of various types of cancers. Recently, it was shown that R-roscovitine could directly inhibit NF- $\kappa$ B at late time-points in cancer cell-lines [25], a finding that, if applicable to neutrophils, might provide insight into the anti-inflammatory properties of R-roscovitine. In this paper we show that R-roscovitine can overcome powerful pro-survival signals to promote neutrophil apoptosis, without directly affecting activation status or key signalling pathways, but by down-regulating the important pro-survival protein Mcl-1 at the level of transcription.

## Results

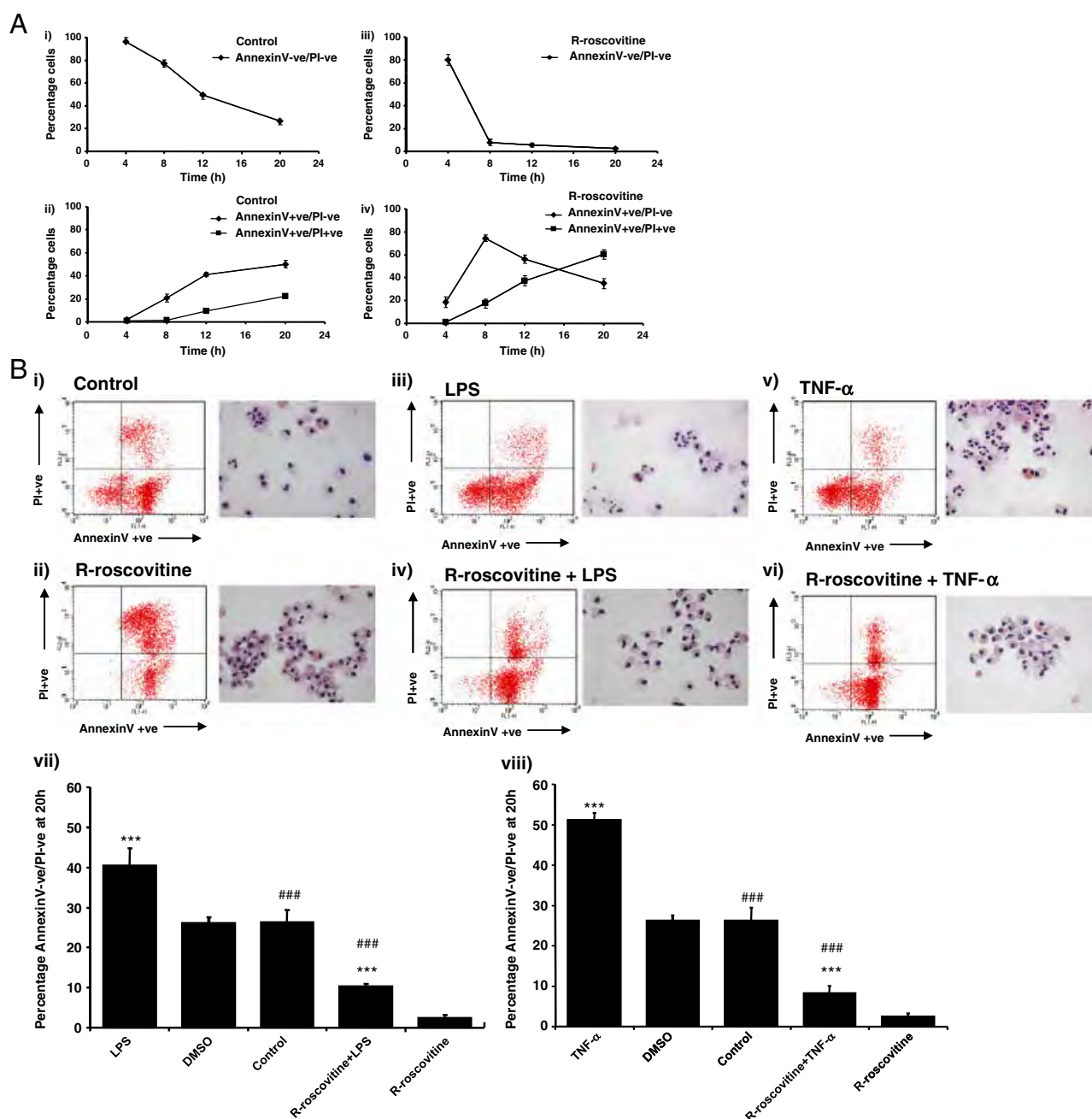
### R-roscovitine promotes a rapid induction of neutrophil apoptosis *in vitro*

R-roscovitine promotes significant classical apoptosis in neutrophils by 4–8 h (Fig. 1a). The majority of neutrophils are apoptotic by 8 h ( $75 \pm 2.7\%$ ) and by 20 h secondary necrosis ( $60.5 \pm 2.1\%$ ) is evident. Time-course experiments are shown to demonstrate that cells transition through apoptosis to necrosis, indicating that secondary as opposed to primary necrosis is induced. Time-lines showing constitutive fate of neutrophils (Fig. 1A), in our laboratory using our methodology, are shown for comparison given the considerable heterogeneity of such data in the literature.

### R-roscovitine over-rides survival mediators TNF- $\alpha$ and LPS to augment neutrophil apoptosis

TNF- $\alpha$  in contrast to its early pro-apoptotic effects is known to promote neutrophil survival at late time-points [7, 26]. LPS is also known to promote neutrophil longevity [27]. In order to investigate the efficacy of R-roscovitine in the presence of





**Figure 1.** (A) Time-course showing effect of R-roscovitine on neutrophil viability over 20 h. Neutrophils were cultured at  $5 \times 10^6$ /mL in IMDM containing 10% autologous serum and R-roscovitine at a concentration of 20  $\mu$ M. At 4, 8, 12 and 20 h samples of cultured neutrophils were removed for generation of cytocentrifuge preparations for examination of morphological changes associated with apoptosis and for preparation of samples for flow cytometric analysis of Annexin V binding and PI staining. Data are presented as a time-course showing the change in untreated neutrophil viability (defined as Annexin V-ve/PI-ve) in (i), apoptosis (Annexin V+ve/PI-ve) and necrosis (Annexin V+ve/PI+ve) both depicted in (ii) over a 20 h time period. The effect of R-roscovitine on the various populations as defined above is shown in (iii) and (iv). Lines showing development of apoptosis and necrosis are superimposed to demonstrate that necrosis follows apoptosis (secondary necrosis). (B) Effect of R-roscovitine on LPS and TNF- $\alpha$  induced neutrophil survival. Neutrophils were cultured at  $5 \times 10^6$ /mL in IMDM containing 10% autologous serum alone as control (i) or with R-roscovitine (20  $\mu$ M) (ii), LPS (100 ng/mL) (iii), R-roscovitine+LPS (iv), TNF- $\alpha$  (10 ng/mL) (v), or R-roscovitine+TNF- $\alpha$  (vi) as indicated above for 20 h. Apoptosis was assessed using Annexin V, PI staining and confirmed by morphological examination under light microscopy. Results represent  $n = 6$ , with each experiment being performed in triplicate. Representative flow cytometry plots (lower left quadrant is annexin V-ve/PI-ve, lower right is annexin V+ve/PI-ve and upper right is annexin V+ve/PI+ve) are shown along with examples of neutrophil morphology from the appropriate experimental condition. Plots show effect of R-roscovitine on neutrophil viability in the presence of LPS (vii) and TNF- $\alpha$  (viii). Statistically significant difference to  $p < 0.001$  between LPS/TNF- $\alpha$  and R-roscovitine+LPS/R-roscovitine+TNF- $\alpha$  is shown as \*\*\*. Statistically significant difference to  $p < 0.001$  between R-roscovitine+LPS/R-roscovitine and control is shown as ### (ANOVA with a Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval).

these pro-inflammatory mediators we cultured neutrophils with R-roscovitine  $\pm$  LPS or TNF- $\alpha$ . Even in the presence of these powerful pro-inflammatory, anti-apoptotic mediators, R-roscovitine significantly reduced neutrophil viability by promoting apoptosis. Viability data and representative examples of neutrophil morphology are shown along with flow cytometry profiles (Fig. 1B).

### **R-roscovitine does not directly activate NF- $\kappa$ B and has no impact on LPS or TNF- $\alpha$ -driven activation of NF- $\kappa$ B**

R-roscovitine does not directly activate NF- $\kappa$ B as assessed by Western blotting for the cytosolic, physiological NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  (Fig. 2A). This is demonstrated by the presence of I $\kappa$ B $\alpha$  in R-roscovitine-treated neutrophil lysate (lane 4). In addition, R-roscovitine does not interfere with the ability of LPS or TNF- $\alpha$  to activate the NF- $\kappa$ B pathway, demonstrated by the loss of I $\kappa$ B $\alpha$  in neutrophil lysates treated with these mediators (lanes 5 and 6, respectively, and compare with mediators alone in lanes 2 and 3). Gliotoxin is a known NF- $\kappa$ B inhibitor and is included for comparison (lanes 7–9); gliotoxin completely inhibited the loss of I $\kappa$ B $\alpha$  induced by LPS and TNF- $\alpha$ .

### **R-roscovitine does not affect p65 translocation stimulated by TNF- $\alpha$ or LPS**

NF- $\kappa$ B activation was investigated by observing with confocal microscopy, the translocation of a green fluorescent-labelled NF- $\kappa$ B subunit, p65, from the cytoplasm to the red, PI-stained nucleus. The translocation of p65, which is representative of NF- $\kappa$ B activation, makes the nucleus appear yellow (Fig. 2B). This method was utilized to ensure that NF- $\kappa$ B activation was not occurring independently of I $\kappa$ B $\alpha$  degradation as has been previously reported [28]. Neutrophils treated with R-roscovitine have a green cytoplasm and a red nucleus, an appearance that contra-indicates activation. However, when neutrophils were co-incubated with R-roscovitine and either TNF- $\alpha$  or LPS the nucleus turned yellow, indicating translocation of the p65 subunit. These confocal microscopy images support our immunoblotting results and indicate that the anti-inflammatory action of R-roscovitine is not mediated by direct inhibition of NF- $\kappa$ B.

### **R-roscovitine has no major effect on ERK phosphorylation**

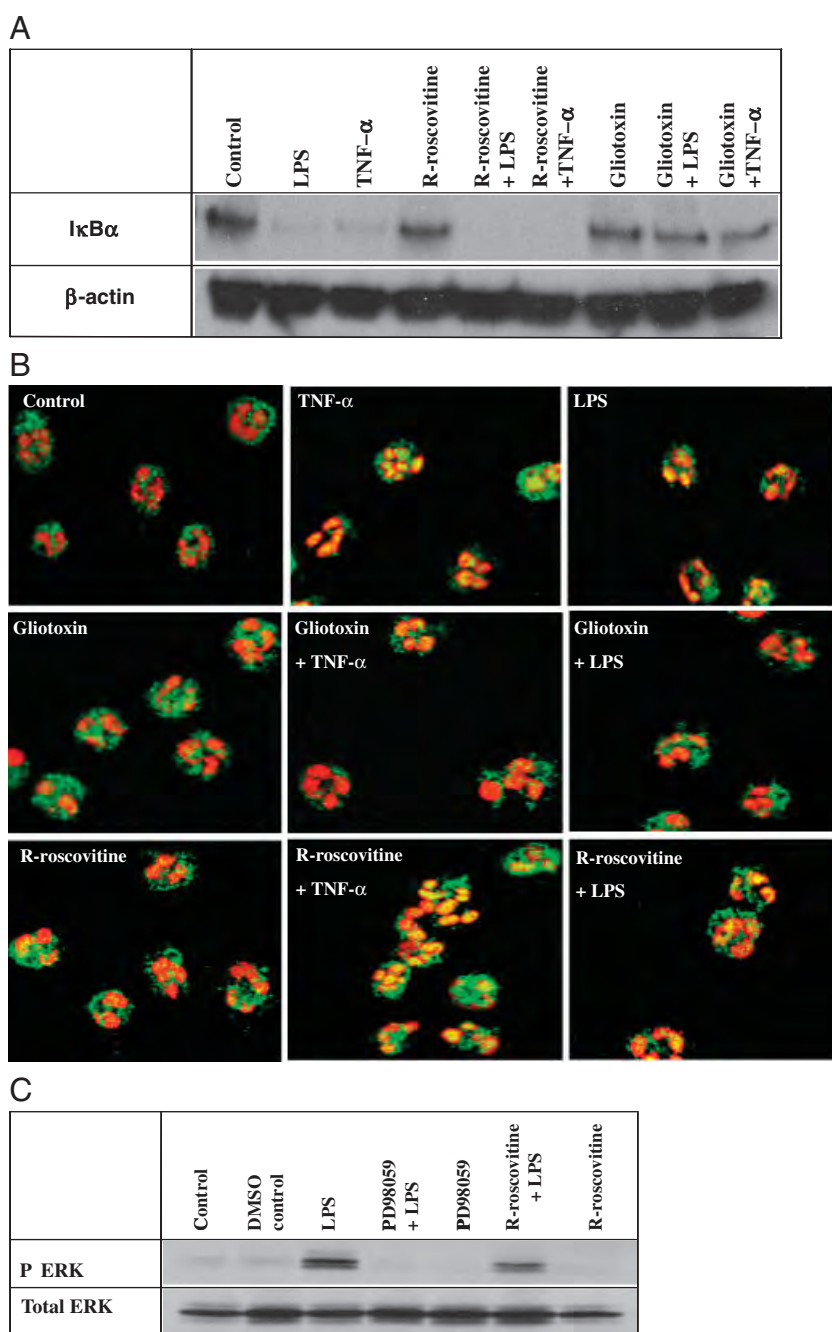
Neutrophils from healthy volunteer donors were incubated with R-roscovitine alone, PD98059 (an ERK-inhibitor) alone or co-incubated with LPS. Neutrophils were cultured with the relevant agents for 4 h. Western blotting reveals complete inhibition of LPS-induced ERK phosphorylation by PD98059 whereas R-roscovitine has a much smaller effect on LPS-induced ERK phosphorylation (Fig. 2C).

### **R-roscovitine has no effect on markers of neutrophil activation**

Neutrophil activation status can determine longevity; so it was important to determine whether R-roscovitine could directly influence the activation status of the cell [16]. Therefore, the effects of R-roscovitine on the cell surface markers CD11b, CD62L and on neutrophil shape change were investigated by flow cytometry. As shown in Fig. 3A, R-roscovitine has virtually no effect on neutrophil activation as assessed by these parameters. On comparison of control samples to R-roscovitine, CD62L shedding (average geometric mean fluorescence value) only altered from 377 to 298, while CD11b up-regulation (average geometric mean fluorescence values) only altered from 40 to 41. Neutrophil shape change as assessed by shift in forward scatter changed from 9 to 16. In addition, R-roscovitine did not affect activation mediated by GM-CSF, LPS, leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and TNF- $\alpha$  when compared with control conditions (Fig. 3A). In contrast, incubation with gliotoxin caused significant alterations in CD11b and CD62L expression and shape change (data not shown). Complementary to this data we have also shown that neutrophil aggregation stimulated by the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or the phorbol ester PMA is unaffected by treatment with R-roscovitine (Fig. 3B). Additionally, R-roscovitine, in contrast to the protein kinase C inhibitor, Ro-31,8220 [29], had no significant effect on the ability of PMA to stimulate production of superoxide anion as measured by intracellular oxidation of dihydrorhodamine (DHR) by flow cytometry (Fig. 3C). In this assay it was also noted that neutrophil shape change as assessed by forward scatter was unaffected by R-roscovitine. It can be concluded that there is no direct effect of R-roscovitine on multiple, pertinent markers of neutrophil activation.

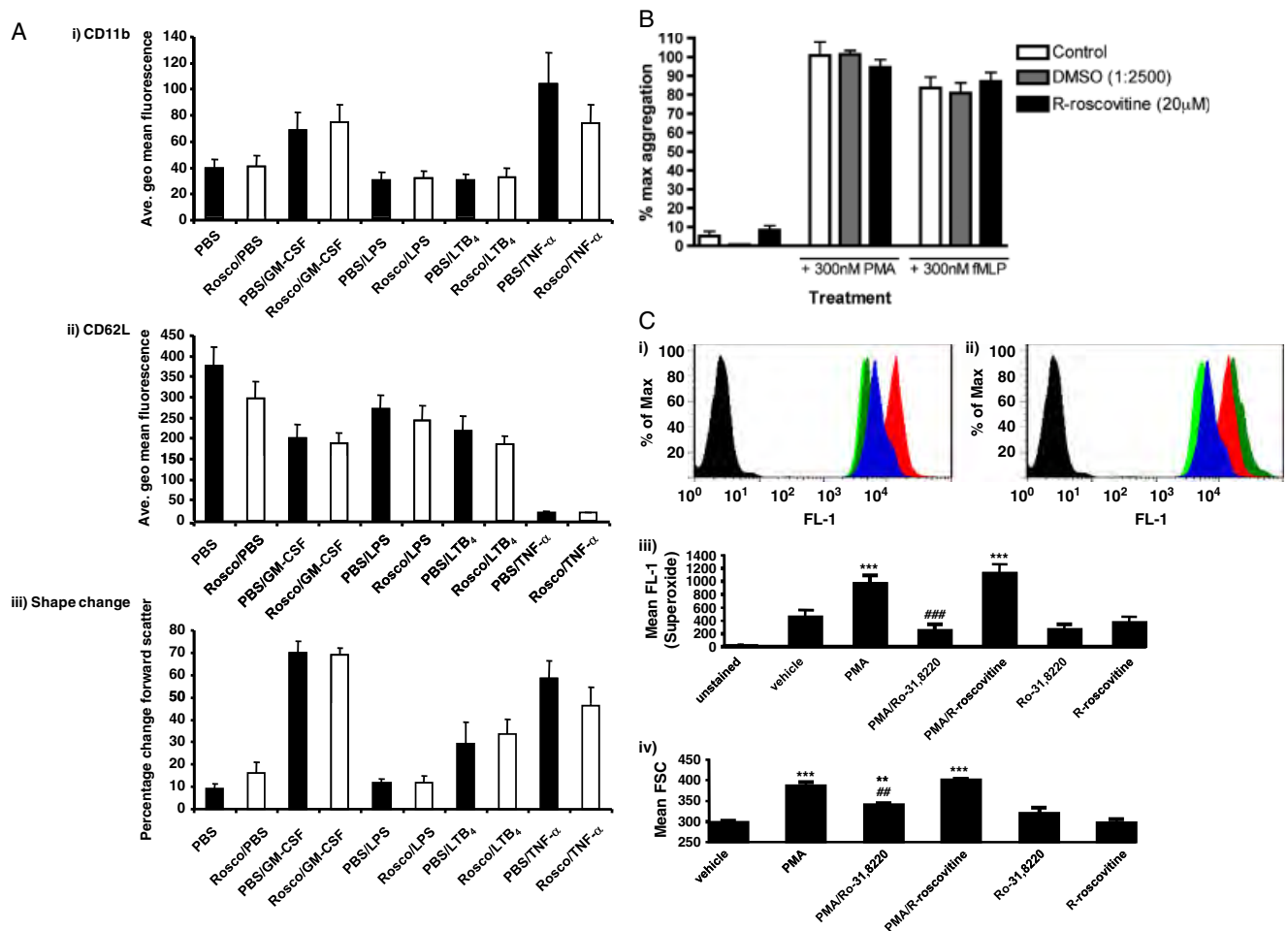
### **R-roscovitine down-regulates Mcl-1 even in the presence of survival factors**

We confirm results previously published by Edwards and colleagues that show levels of Mcl-1 protein decline by 4 h and that the cytokine GM-CSF has a protective effect against Mcl-1 decline (Fig. 4A) [18]. We subsequently demonstrated that R-roscovitine augmented the decrement in levels of Mcl-1 in neutrophils at 4 h (effect also evident at 2 h; results not shown) and that it had the ability to over-ride the protective effect of LPS, TNF- $\alpha$  and GM-CSF on Mcl-1 (Fig. 4A). However, when R-roscovitine was co-incubated with TNF- $\alpha$ , levels of Mcl-1 were reduced more substantially than with R-roscovitine alone (Fig. 4A). No consistent pattern was discernable when the same experiment was performed probing for XIAP protein levels. We believe that this occurred because changes in levels of XIAP appear very close to the final stages of apoptosis and this physiological event makes interpretation of these results difficult.



**Figure 2.** (A) Effect of R-roscovitine on I $\kappa$ B $\alpha$  degradation in neutrophils. Neutrophils ( $5 \times 10^6$ /mL in IMDM containing 10% autologous serum) were treated in either the absence (control) or presence of R-roscovitine (20  $\mu$ M) or gliotoxin (0.1  $\mu$ g/mL) for 30 min following which, control (IMDM+10% autologous serum), LPS (100 ng/mL) or TNF- $\alpha$  (10 ng/mL) treatments were added to each of the previous conditions for a further 30 min. The blots were probed for I $\kappa$ B $\alpha$ . Blot shown is representative of three experiments. (B) Effect of R-roscovitine on p65 translocation. Neutrophils ( $2.5 \times 10^6$ /mL in IMDM containing 10% autologous serum) were treated in either the absence (control) or presence of R-roscovitine (20  $\mu$ M) or gliotoxin (0.1  $\mu$ g/mL) for 30 min following which, control (IMDM+10% autologous serum), LPS (100 ng/mL) or TNF- $\alpha$  (10 ng/mL) treatments were added to each of the previous conditions for a further 30 min. The nuclei were stained using PI and the movement of green fluorescence-labelled p65 was assessed using confocal microscopy. The confocal images show p65 as green fluorescence, nuclei as red fluorescence and yellow fluorescence indicates colocalization. (C) Effect of R-roscovitine on LPS induced ERK activation. Neutrophils at  $5 \times 10^6$ /mL were incubated in IMDM containing 10% autologous serum alone or with the addition of DMSO (Vehicle control), LPS (100 ng/mL), PD98059 (50  $\mu$ M), R-Roscovitine (20  $\mu$ M) or combinations of these as designated above. After 4 h incubation they were lysed and immunoblotted for total and phosphorylated ERK. Blot shown is representative of three similar experiments.





**Figure 3.** (A) Changes in expression of CD11b, CD62L and shape change. Neutrophils at  $5 \times 10^6$ /mL in PBS were treated in the absence (control) or presence of R-roscovitine (20  $\mu$ M) for 30 min and then with either PBS (control), GM-CSF (50 ng/mL), LPS (100 ng/mL), leukotriene B<sub>4</sub> (1  $\mu$ M) or TNF- $\alpha$  (10 ng/mL) for a further 30 min. Samples were then labelled with antibodies for CD11b, CD62L or the control antibodies APC and PE. Shape change was assessed by measurement of change in forward scatter. Labelling was assessed by flow cytometry and all experiments were performed at least three times. There was no statistically significant difference between R-roscovitine-treated cells and control ( $p > 0.1$  ANOVA with a Student Newman-Keuls multiple comparison *post hoc* test with a 95% confidence interval). (B) Changes in neutrophil aggregation. Neutrophils ( $5 \times 10^6$  cells/mL; 500  $\mu$ L) were pretreated with R-roscovitine (20  $\mu$ M), DMSO (1:2500) or control buffer for 7 min then stimulated by PMA (300 nM) or fMLP (300 nM) and aggregation assessed after a further 7 min. Data are expressed as percentage of maximum aggregation induced by either PMA or fMLP using a four-channel optical aggregometer (Chronolog, Labmedics, UK). Results are mean  $\pm$  SEM of  $n = 4$  separate experiments (ANOVA with a Student Newman-Keuls multiple comparison *post hoc* test with a 95% confidence interval). (C) Changes in superoxide production. Neutrophils at  $1 \times 10^6$ /mL in HBSS were pre-treated with R-roscovitine (20  $\mu$ M), Ro-31,8220 (1  $\mu$ M) or vehicle (0.0004% DMSO) control prior to stimulation of superoxide anion generation (i–iii) and shape change (iv) by PMA (300 nM) for 15 min. Representative overlay flow-cytometric plots for DHR fluorescence (FL-1) are depicted in (i) and (ii). In (i), unstained (black), vehicle (blue), PMA (red), Ro-31,8220 (light green) and Ro-31,8220 plus PMA (dark green). In (ii), unstained (black), vehicle (blue), PMA (red), R-roscovitine (light green) and R-roscovitine plus PMA (dark green). For comparison, unstained, vehicle and PMA plots are the same in (i) and (ii). In panels (iii) data are mean FL-1  $\pm$  SEM of  $n = 4$  separate donors with each sample done in duplicate for each experiment where \*\*\* represents  $p < 0.001$  versus vehicle control and ## represents  $p < 0.001$  versus PMA alone. In panel (iv), data are mean FSC  $\pm$  SEM of  $n = 4$  separate donors with each sample done in duplicate for each experiment where \*\*\* and \*\* represent  $p < 0.001$  and  $p < 0.01$  versus vehicle control, respectively, and # represents  $p < 0.01$  versus PMA alone (ANOVA with a Student Newman-Keuls multiple comparison *post hoc* test with a 95% confidence interval).

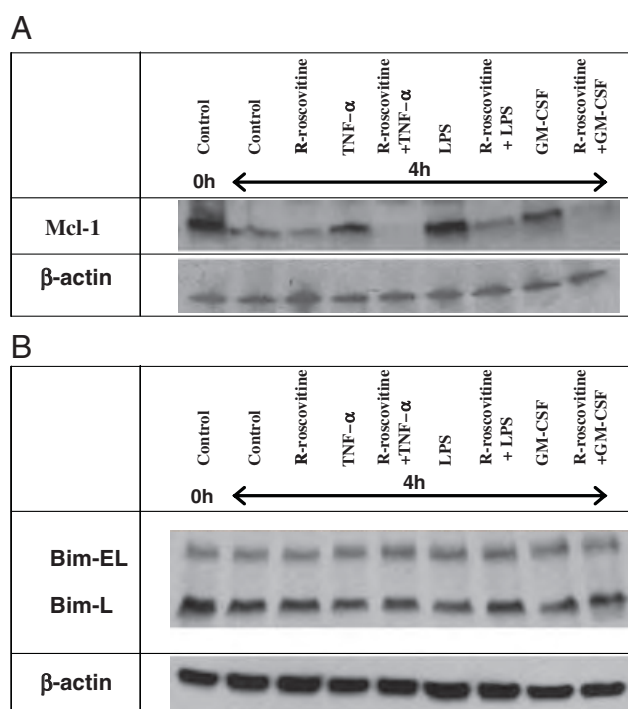
### R-roscovitine has no effect on the key pro-apoptotic Bcl-2 homologue Bim

R-roscovitine has no effect on the protein levels of Bim. Neutrophils treated for 4 h in the presence or absence of the survival factors LPS, TNF- $\alpha$  and GM-CSF maintained constant levels of Bim long and Bim extra long (Fig. 4B). It can be concluded that R-roscovitine either directly or indirectly down-regulates two key neutrophil survival proteins without affecting

the pro-apoptotic protein Bim, tipping the delicate balance of viability towards apoptosis.

### R-roscovitine down-regulates Mcl-1 at the mRNA level

We hypothesized that R-roscovitine was mediating its effects on neutrophil survival by influencing production of key survival proteins at the level of transcription. We have shown this with



**Figure 4.** (A) Effect of R-roscovitine on the survival protein Mcl-1. Neutrophils at  $5 \times 10^6/\text{mL}$  in IMDM containing 10% autologous serum were either lysed immediately (time 0) or incubated in either the absence (control) or presence of GM-CSF (50 ng/mL), LPS (100 ng/mL), TNF- $\alpha$  (10 ng/mL), R-roscovitine (20  $\mu\text{M}$ ), R-roscovitine+GM-CSF, R-roscovitine+LPS or R-roscovitine+TNF- $\alpha$ , gliotoxin (0.1  $\mu\text{g}/\text{mL}$ ), R-roscovitine+gliotoxin for 4 h and then lysed and immunoblotted for Mcl-1 or  $\beta$ -actin. Blot shown is representative of three experiments. (B) Effect of R-roscovitine on the pro-apoptotic bcl-2 homologue Bim. Neutrophils treated as in Fig. 4A and probed with anti-Bim antibody. Blot shown is representative of three experiments.

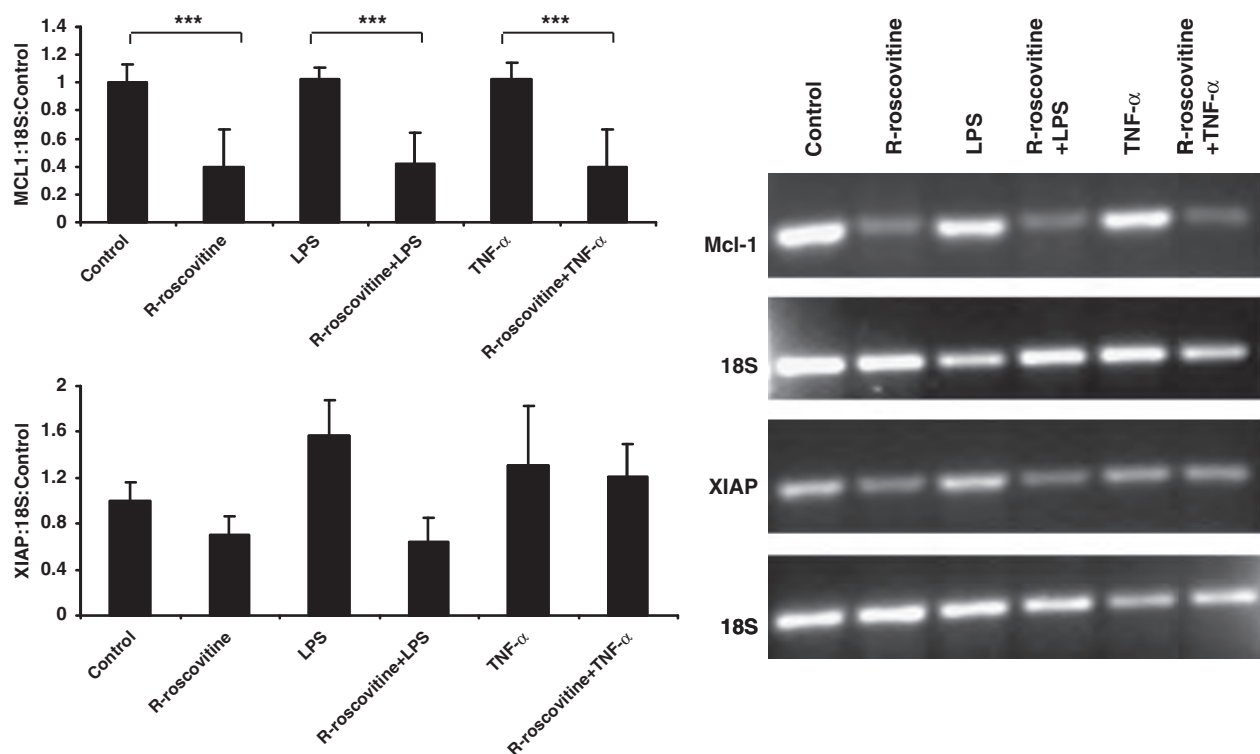
the use of semi-quantitative PCR. R-roscovitine down-regulates Mcl-1 at the mRNA level even in the presence of survival factors such as LPS and TNF- $\alpha$  (Fig. 5). By contrast, R-roscovitine did not significantly affect basal levels of XIAP mRNA at 4 h but did seem to prevent its up-regulation by LPS (not quite statistically significant when all samples were analysed as *per methods*). TNF- $\alpha$  did not appear to up-regulate transcription of XIAP.

## Discussion

R-roscovitine is a 2,6,9-substituted purine analogue that, in common with other CDK-inhibitors, competes for the ATP-binding site on CDK [30]. CDK are important regulators of cell-cycle progression but also gene transcription [31]. R-roscovitine has been extensively evaluated as a cancer therapeutic both independently and in combination with other drugs [31, 32]. We have shown previously that R-roscovitine not only promotes neutrophil apoptosis *in vitro* but does so in the presence of powerful survival factors [23]. This is despite the fact that neutrophils are terminally differentiated cells and were

thought not to have functionally active CDK. R-roscovitine also dramatically enhanced the resolution of established animal models of neutrophil-dominant inflammation such as: carrageenan elicited acute pleurisy, bleomycin lung injury and passively-induced arthritis. Peripheral neutrophil counts were unaffected indicating a reassuring specificity for stimulated inflammatory neutrophils [23]. Interestingly, in a recent study R-roscovitine has been used to induce neutrophil apoptosis *in vitro* and *in vivo* and shown to resolve inflammation after pneumococcal meningitis and accelerate recovery in mice treated with antibiotics [33]. The molecular mechanism by which R-roscovitine achieves these promising results has not yet been elucidated. R-roscovitine is known to promote apoptosis in cancer cell lines by inhibition of RNA polymerase-II leading to down-regulation of the key survival protein Mcl-1 [24]. It has also been suggested that NF- $\kappa\text{B}$  activation at the level of I $\kappa\text{B}$  kinase activity is inhibited by R-roscovitine [25]. Given that both Mcl-1 and NF- $\kappa\text{B}$  are key components of neutrophil survival regulation we were keen to address the hypothesis that R-roscovitine might act *via* similar mechanisms in the inflammatory neutrophil. We propose that down-regulation of the survival protein Mcl-1 at the level of transcription is responsible for the pro-apoptotic action of R-roscovitine. We show that this effect is counter-intuitively enhanced in the presence of powerful survival mediators such as GM-CSF, LPS and TNF- $\alpha$ . However, despite the evidence that R-roscovitine may directly inhibit NF- $\kappa\text{B}$  activation in some cancer cell lines we could find no evidence of this in resting or stimulated neutrophils. We performed dual experiments, I $\kappa\text{B}\alpha$  Western blotting and confocal microscopy for labelled p65, because recent reports had suggested that NF- $\kappa\text{B}$  activation could occur independently of I $\kappa\text{B}\alpha$  inhibition [28, 34]. We also examined mRNA and protein levels of XIAP, an NF- $\kappa\text{B}$ -regulated survival protein. We could achieve no consistent results immunoblotting for XIAP, perhaps because XIAP acts in the final stages of apoptosis and results are confounded by the conclusion of this process. We feel that in our system down-regulation of XIAP was more consistent with concluded apoptosis. Additionally, we have shown that ERK activation induced by LPS is minimally affected by R-roscovitine. This finding is important as it suggests that the documented pharmacological activity of R-roscovitine against ERK 1 and 2 is not relevant to neutrophil apoptosis.

Despite failing to confirm a role for R-roscovitine-induced inhibition of NF- $\kappa\text{B}$  activation in neutrophils, as suggested by a recent study of cancer cell-lines, we did find that R-roscovitine significantly down-regulated Mcl-1. We demonstrated enhanced activity in the presence of the survival agents LPS and TNF- $\alpha$ , although only an attenuation of the survival effect of GM-CSF was seen. Mcl-1 is extensively regulated and has been associated with various signalling systems including PI3K, MAPK and glycogen synthase kinase [21, 35]. It can be down-regulated at the level of transcription, translation and even degradation [17]. Mcl-1 may be degraded in the proteasome following phosphorylation and ubiquitination or may be disposed of in a caspase-dependent manner [17–20]. It is also possible that a splice variant of Mcl-1 has pro-apoptotic capability, which may perhaps



**Figure 5.** PCR for XIAP and MCL-1. RNA extracted from neutrophils treated for 2 h (MCL-1) and 4 h (XIAP) with treatments as designated at the same concentrations as described in Fig. 4A. Gels shown are representative and densitometry is from three experiments. Statistically significant difference for MCL1 between R-roscovitine and control, LPS and R-roscovitine/LPS, TNF- $\alpha$  and R-roscovitine/TNF- $\alpha$   $p < 0.001$  \*\*\* (ANOVA with a Student Newman-Keuls multiple comparison *post hoc* test with a 95% confidence interval).

explain the rapid apoptosis that can accompany Mcl-1 down-regulation.

We felt it was important to consider the effect of R-roscovitine on neutrophil activation status. Agents that cause neutrophil priming render them susceptible to activation. Ultimately, this may lead to release of superoxide anion and other toxic products detrimental to the process of resolution of inflammation. The effects of R-roscovitine on the markers of activation CD11b (which becomes up-regulated upon activation by granule movement to the surface membrane), CD62L (which is shed upon priming/activation) and neutrophil shape change (neutrophils when stimulated re-organize their cytoskeleton resulting in cell polarization) were investigated using flow cytometry. Similarly, neutrophil aggregation was assessed by measurement of light transmission through populations of stimulated neutrophils. Results demonstrated that R-roscovitine caused no significant alteration in the activation status of neutrophils on its own or in combination with neutrophil-activating stimuli, a promising finding when considering its potential therapeutic use as a novel anti-inflammatory agent. Additionally, the ability of neutrophils to generate superoxide anion in response to stimulation was not compromised by treatment with R-roscovitine. The preservation of neutrophil activation status in co-treatments of R-roscovitine and the stimuli GM-CSF, LPS, leukotriene B<sub>4</sub> and TNF- $\alpha$  also infers important information regarding the recruitment and migration of neutrophils from the circulation to sites of inflam-

mation as the neutrophil surface molecules CD11b and CD62L are important regulators of cell adhesion to vascular endothelium. It is pertinent that neutrophils are recruited to sites of infection and inflammation, as dysregulation in this process is associated with increased susceptibility to infection [36].

It was previously shown that guanosine triphosphate (GTP)-dependent secretion in the neutrophil is regulated by CDK5 and that R-roscovitine blocks lactoferrin secretion and reduces expression of CD63 and CD66b on the surface membrane. This implies that CDK5-p35 activity is required for optimum levels of GTP-induced secretion from primary (azurophil) and secondary (specific) granules [37, 38]. We found no change in levels of CD11b localization (which resides in secondary granules) upon treatment with R-roscovitine when compared with control. This could be because GTP-induced secretion regulated by CDK5-p35 activity is redundant or it may reflect sub-optimal inhibition of CDK5 by R-roscovitine. Only partial inhibition occurred in previous studies, indicating that alternative mechanisms are capable of compensating.

We have also shown that R-roscovitine has a much smaller effect than the ERK-specific inhibitor PD50890 on LPS-induced ERK phosphorylation. This contrasts with work on vascular smooth muscle cells where R-roscovitine inhibited ERK activation by angiotensin II [39]. It seems likely that the early, dramatic effects of R-roscovitine on neutrophil survival are unlikely to be due to this sub-optimal inhibition of ERK phosphorylation. We feel that

the observed effect is more likely to be due to downstream effects of R-roscovitine as opposed to direct inhibition especially as even powerful inhibition of ERK phosphorylation does not induce neutrophil apoptosis [14]. We have shown that R-roscovitine induces neutrophil apoptosis and also that it can resolve animal models of inflammation [23]. Interestingly, the ERK inhibitor PD50890 was also shown to have a pro-resolution effect in carrageenan-induced pleurisy without having a direct effect on neutrophil apoptosis *per se* [14]. Pro-resolution effects were felt to be conferred by inhibition of inflammatory mediator-triggered ERK activation responsible for neutrophil survival. Given that ERK 1 and 2 fall within the pharmacological specificity of R-roscovitine it was possible that ERK inhibition could account for some of its effects [31]. However, our findings suggest that ERK inhibition is not relevant to CDK-inhibitor-mediated resolution of inflammation. R-roscovitine allows LPS and TNF- $\alpha$ -mediated activation of both NF- $\kappa$ B and ERK and yet overcomes the survival signalling usually associated with these pathways. This suggests that the pro-apoptotic effect of R-roscovitine occurs down-stream of these events and is of sufficient magnitude to over-ride them. In keeping with this hypothesis we have shown that the key survival protein Mcl-1 is significantly down-regulated.

We have shown that Mcl-1 is down-regulated at the mRNA level by R-roscovitine and this change is readily detected at 2 h and is more pronounced at 4 h. We believe that MCL-1 down-regulation precedes and is responsible for the initiation of apoptosis because we detect changes in the level of this mRNA/protein at 2 h but only detect apoptosis by annexin-V binding at 4 h and caspase cleavage at 2.5–3 h (Western blotting and fluorometric caspase assay, data not shown). By contrast R-roscovitine does not appear to decrease basal levels of XIAP but there was a trend toward prevention of up-regulation by LPS at the mRNA level. These data are not as clear-cut as the Mcl-1 findings, possibly because the half-life of XIAP mRNA is significantly longer than that of MCL-1 [40]. The interpretation of results for TNF- $\alpha$  is complicated but interesting because of the dual and directly opposing effects of TNF- $\alpha$  on neutrophil apoptosis [7, 21]. TNF- $\alpha$  both directly activates the extrinsic apoptotic program and enhances transcription of key survival proteins *via* the NF- $\kappa$ B pathway. The combination of R-roscovitine and TNF- $\alpha$  leads to very early initiation of and bias towards the extrinsic apoptotic program detectable by annexin-V binding at 2 h (data not shown). XIAP is up-regulated and stabilized in response to caspase cleavage; so it might be expected that we would find enhanced mRNA/protein levels of XIAP. However, there is no detectable up-regulation as measured by PCR, which probably represents a degree of up-regulation in response to the early initiation of apoptosis that is balanced by inhibition of transcription by R-roscovitine. We have shown that *de novo* transcription of XIAP as stimulated by LPS is prevented by R-roscovitine.

By demonstrating that the important survival protein Mcl-1 is down-regulated at transcriptional level, we corroborate our finding that direct inhibition of NF- $\kappa$ B is not responsible for CDK-inhibitor-driven neutrophil apoptosis. We hypothesize that a more central, generalized effect on neutrophil transcription is responsible for the induction of apoptosis. This effect is particularly evident in granu-

locytes because of the importance of rapidly transcribed and degraded, short-lived protein Mcl-1 to their survival. Pro-apoptotic Bcl-2 homologues such as Bim have much longer half-lives and in excess drive neutrophil apoptosis *via* the intrinsic (mitochondrial) pathway. Our work adds to the literature, suggesting the fundamental importance of these proteins to neutrophil survival and further illuminates the mechanism of action of R-roscovitine.

It can be concluded from this study that R-roscovitine induces apoptosis in human neutrophils by decreasing levels of pro-survival proteins to over-ride the cyto-protective effects of inflammatory mediators without directly influencing key inflammatory signalling pathways. In addition, data provided enhance the claim that R-roscovitine would be well tolerated *in vivo* (corroborated by the use of R-roscovitine in clinical trials as an anti-cancer agent) and provide support for a therapeutic trial of R-roscovitine in neutrophil-dominant inflammatory disease.

## Materials and methods

### Reagents

Hanks balanced salt solution (HBSS) and IMDM were obtained from PAA. Dextran T500 obtained from Pharmacosmos, Percoll and ECL Western blotting detection reagents were obtained from GE Healthcare. AnnexinV-fluos from Roche and PI from Sigma-Aldrich. Recombinant human TNF- $\alpha$  and human GM-CSF from R&D Systems. R-roscovitine, PD98059 and LT-B<sub>4</sub> were from Merck, Dexamethasone was obtained from Organon. LPS (*E. Coli* serotype O127:B8), Gliotoxin, PMA (phorbol 12-myristate 13-acetate) and fMLP from Sigma-Aldrich, DHR from Invitrogen and Ro-31,8220 from Calbiochem. XIAP primers from eurofins (XIAP forward gga acc ttg tga tcg tgc ct, XIAP reverse aat cag tta gcc ctc ctc ca). Mcl-1 primer pair commercially available from R&D Systems (See: <http://www.rndsystems.com/>), 18S competimers from Applied Biosystems and PCR Master-mix purchased from Promega, GelRed from Biotium.

### Antibodies

Anti-Mcl-1, Anti-XIAP and Anti-Bim antibodies were purchased from Santa Cruz, R&D Systems and Cell Signalling, respectively. Anti- I $\kappa$ B $\alpha$  was from Abcam. Monoclonal anti- $\beta$ -actin antibody was from Sigma-Aldrich. Goat anti-mouse and -rabbit HRP-conjugated antibodies were obtained from Dako. Anti-CD62L-PE and anti-CD11b-APC from BD Bioscience, anti-p65 primary antibody from (R&D Systems).

### Cell isolation and culture

Polymorphonuclear leukocytes were isolated from peripheral blood of healthy donors as described previously [7]. Polymorphonuclear

leukocytes were >95%; neutrophils assessed using morphologic criteria and cell viability (>99%) was assessed by trypan blue exclusion. Cells were re-suspended at  $5 \times 10^6$ /mL in IMDM plus 10% autologous serum (apart from where otherwise stated) and treated in 2 mL Eppendorf tubes at 37°C on a shaking heat block or cultured in flat-bottomed flexible well plates in a 5% CO<sub>2</sub> atmosphere at 37°C.

### Assessment of granulocyte apoptosis

Neutrophil apoptosis was assessed by cyto-centrifuging 100 µL of  $5 \times 10^6$  cells/mL at 300 rpm for 3 min. The cells were then fixed for 1 min in methanol and stained with Diff-Quik™ (Gamidor). Morphology was assessed at 100× objective on an oil immersion microscope. Additionally FITC-labelled rh-annexin V and PI were used to assess apoptosis and necrosis, respectively. Annexin V was diluted 1/500 in binding buffer (HBSS+Ca<sup>2+</sup>) and 280 µL added to 20 µL of cells ( $5 \times 10^6$  cells/mL). Samples were then incubated on ice at 4°C for 10 min. Immediately prior to processing on the FACSCaliber flow cytometer, 1 µL of PI (1 mg/mL) *per* sample was added.

### Assessment of CD62L, CD11b and shape change

Neutrophils were resuspended at  $5 \times 10^6$ /mL in PBS+cations and treated with reagents as detailed in the appropriate figure legend. Treatments took place on a 37°C shaking heat block; 50 µL of cells were then added to 150 µL ice cold PBS without cations containing 0.1% BSA and treated with 2 µL of either the control PE/APC antibodies or with antibodies for CD62L and CD11b for 30 min prior to assessment by flow cytometry.

### Measurement of reactive oxygen species generation

Superoxide anion generation was estimated as an increase in reactive oxygen species as determined by DHR fluorescence. Neutrophils were resuspended at  $2 \times 10^6$  cell/mL in HBSS medium and kept at 37°C. Cells were pre-incubated with DHR (1 µM) for 5 min then diluted to  $1 \times 10^6$ /mL for the treatment with R-roscovitine (20 µM) or the protein kinase C inhibitor Ro-31,8220 (1 µM) for 30 min. Cells were then stimulated by the addition of PMA (300 nM) for a further 15 min and placed on ice. Changes in mean fluorescence (FL-1) and mean forward scatter were measured by flow cytometry to determine superoxide anion generation and shape change, respectively.

### Neutrophil aggregation

Neutrophils were prepared at  $5 \times 10^6$  neutrophils/mL in HBSS medium. Analysis of aggregation was performed using a four-

channel optical aggregometer (Chronolog, Labmedics) and data were captured using an analogue-digital converter (MacLab 4c, AD Instruments).

### Western blotting

Cells at a concentration of  $5 \times 10^6$ /mL *per* condition were incubated at 37°C on a shaking heat block. For times and reagent concentrations please refer to figure legends. Lysates, prepared as described previously [7], were run on a 12% SDS gel and transferred onto PVDF (Immobilon-P, Millipore). Membranes were blocked for 1 h in 5% wt/vol dried milk/TBS/0.1% Tween-20 or 5% wt/vol BSA/TBS/0.1% Tween-20 (Anti-Bim) prior to overnight incubation at 4°C (or 1.5 h at room temperature for β-actin) with primary antibodies for Mcl-1 diluted 1:200, β-actin diluted 1:10 000, XIAP diluted 1:1000, IκBα diluted 1:2500, Bim 1:1000. Following 3 × 5 min washes in TBS/0.1% Tween-20 the blots were incubated with the appropriate HRP-conjugated secondary antibody diluted 1:2500 for 1 h at room temperature prior to incubation with ECL reagents, exposure to BioMax MS-1 light-sensitive film, and processing through an x-ray developer (X-Ograph Imaging Systems).

### RT-PCR (semi-quantitative)

Total neutrophil RNA was isolated with the use of a Nucleospin RNA II kit (Macherey-Nagel) as *per* the manufacturer's instructions. Two micrograms of RNA were made up to a final volume of 25 µL with DEPC-ddH<sub>2</sub>O. Reaction mix for each sample prepared as follows: 10 µL 5× M-MLV reaction buffer, 5 µL 100 mM DTT, 5 µL 100 µg/mL oligo(dT) primer, 4 µL 10 mM dNTPs, 0.5 µL Rnasin, 0.5 µL M-MLV reverse transcriptase; 25 µL of this mix added to each 2 µg RNA sample prior to incubation for 90 min at 37°C to allow cDNA synthesis. Reaction stopped by incubating at 90°C for 10 min; 2 µL Primers and 5 µL cDNA added to 25 µL Master mix and volume made up to 50 µL with diethylpyrocarbonate H<sub>2</sub>O. Tubes placed in thermal-cycler with following program: 94°C for 4 min then: 94°C for 45 s, 55°C for 45 s, 72°C for 45 s × 30–35 cycles, 72°C for 10 min. Products separated by agarose gel electrophoresis using GelRed nucleic-acid detection dye prior to visualization and image capture with UV-light camera.

### Immunohistochemistry and confocal analysis

Freshly isolated neutrophils at  $2.5 \times 10^6$ /mL were treated as above; 100 µL of sample was centrifuged at 400g, 4°C for 4 min in a flat-bottomed flexi-well plate. Supernatants were removed and cells re-suspended and fixed for 20 min in 3% paraformaldehyde. After washing and quenching with 50 mM glycine, the cells were re-suspended in 50 µL of 10% goat serum and blocked for 1 h. Cells were centrifuged again, supernatant removed and cells

re-suspended and incubated with 50  $\mu$ L of anti-p65 primary antibody diluted 1:50 and 50  $\mu$ L of PI (2  $\mu$ L in 1 mL) for 1 h. Following washing the cells were incubated with 50  $\mu$ L of alexa 488 Fab2 fragment (Invitrogen) 1:500 for 1 h, washed and resuspended at 150  $\mu$ L. Aliquots of 100  $\mu$ L volume were removed and cyto-centrifuged onto slides prior to visualization on confocal microscope (Zeiss LSM510meta).

## Statistical analyses

All experiments were performed at least three times unless stated otherwise and each treatment done in triplicate and the results are expressed as the mean  $\pm$  SEM. Data were analysed by a one-way ANOVA with a Student Newman-Keuls multiple comparison *post hoc* test with a 95% confidence interval (InStat software).

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- Abbreviations:** CDK: cyclin-dependent kinase · DHR: dihydrorhodamine · fMLP: N-formyl-methionyl-leucyl-phenylalanine · GTP: guanosine triphosphate · HBSS: Hanks balanced salt solution · LTB<sub>4</sub>: leukotriene B<sub>4</sub>
- Full correspondence:** Dr. Andrew Leitch, MRC Centre for Inflammation Research, The Queen's Medical Research Institute, University of Edinburgh Medical School, 47 Little France Crescent, Edinburgh, Scotland, UK  
 Fax: +441312426578  
 e-mail: aleitch3@staffmail.ed.ac.uk
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